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Ecología del fitoplancton colonial

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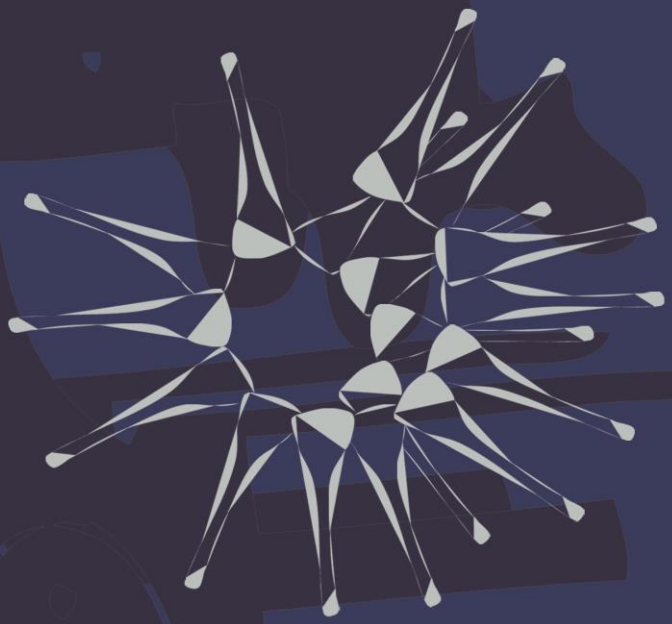

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The ecology of colonial phytoplankton

Ecología del fitoplancton colonial

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Resumen

Introducción

Los orígenes de los organismos que componen la comunidad fitoplanctónica se remontan a distintos eventos endosimbióticos (Falkowski *et al.* 2004), es por ello que una de las principales características del fitoplancton es una enorme diversidad, tanto en sus atributos morfológicos y fisiológicos como en sus formas de vida. Actualmente no se conoce el motivo que favoreció la transición desde la unicelularidad a la colonialidad. La multicelularidad de algunas especies de fitoplancton tendría ventajas para asegurar la conservación de la línea germinal y generar un medio interno estable que proteja a las células que forman la colonia del cambiante ambiente que la rodea (Gerhart & Kirschner 1997). Además, organismos coloniales evolutivamente más avanzados, en los que se observa una diferenciación celular y división de tareas (como es el caso de la volvocal *Volvox*) empezarían a definir una estrategia de vida compleja y jerarquizada. El paso de una forma de vida unicelular a una colonial para un organismo fitoplanctónico conlleva implícito un número de condicionantes ecológicos que determinan cuándo y cómo las formas coloniales pueden ser más viables.

La variedad de formas, tamaños y configuraciones presentadas por las células y colonias nos indica que no hay una estructura óptima, sino un gran abanico de posibilidades exitosas para afrontar los obstáculos a los que estos organismos se enfrentan durante su ciclo de vida. Unicelulares y formas coloniales son ubicuas en el fitoplancton y han perdurado durante millones de años, por ello resulta interesante analizar cuáles son las ventajas ecológicas que confiere la colonialidad y en qué modo han compensado las desventajas derivadas de la agregación para haberse convertido en una alternativa evolutivamente exitosa a la unicelularidad. La supervivencia de estos organismos es el resultado del balance entre la adquisición de los nutrientes necesarios para la replicación celular y la protección ante potenciales depredadores y prevención del hundimiento. En relación con estos procesos de ganancia y pérdida, el tamaño y la forma de los diferentes organismos juegan un papel fundamental, definiendo, en última instancia, las estrategias vitales que determinarán su éxito (o fracaso) ecológico en un ambiente

dado. Esta tesis está encaminada a analizar las circunstancias ecológicas asociadas a una vida colonial en el fitoplancton.

La formación de una colonia implica el aumento del tamaño del organismo. Fueron muchas las investigaciones que a lo largo de la segunda mitad del siglo XX exploraron la relación existente entre el tamaño de los organismos fitoplanctónicos y sus efectos en su fisiología y ecología. Los estudios de Williams (1964) y Eppley and Sloan (1966) encontraron que las tasas específicas de máximo crecimiento (tiempo $^{-1}$) estaban relacionadas negativamente con el volumen celular, hecho que fue confirmado más tarde por otros autores (Banse 1976; Chisholm 1992). Investigaciones posteriores justificaron esta relación negativa entre tamaño y la tasa de crecimiento debido a que la absorción y utilización de los nutrientes era más eficiente en organismos unicelulares por razones de superficie-volumen (Raven 1998), y a que el transporte interno de nutrientes se veía dificultado en organismos de mayor tamaño, debido al aumento en las distancias intracelulares (West *et al.* 1999). Como resultado, se consideró que los organismos fitoplanctónicos de mayor tamaño estaban en desventaja para competir por los nutrientes (Smith & Kalff 1982; Grover 1989) y, por tanto, para sobrevivir en situación de escasez de nutrientes en el medio (Chisholm 1992). Así mismo, las cuotas celulares de nutrientes que un organismo fitoplanctónico necesita para replicarse son directamente proporcionales a su tamaño (Smith & Kalff 1982; Grover 1989) y por lo tanto, los unicelulares de menor tamaño pueden cubrirlos de una forma más sencilla que en el caso de una unicelular más grande o una colonia.

Entre los nutrientes esenciales para el desarrollo de las vidas fitoplanctónicas, el fósforo (P) juega un papel ecológico clave ya que, debido a su habitual escasez, es el recurso que más limita el crecimiento de estos organismos en aguas dulces (Hudson *et al.* 2000). La cantidad de fósforo disponible en el medio limita el flujo del mismo hacia el fitoplancton, y a su vez, este flujo está vinculado con el tamaño celular, como expresa la ley de Fick (Pasciak & Gavis 1974; Jumars *et al.* 1993):

$$Q_P = 4 \pi D r_c (C_\infty - C_0)$$

Donde Q_P es el flujo de nutrientes hacia una célula esférica modelo, D es el coeficiente molecular de difusión del soluto, r_c es el radio celular y $(C_\infty - C_0)$ es la concentración de nutriente en la superficie celular. Por lo tanto, cuando la absorción por unidad de superficie a nivel de membrana es constante, la tasa de

absorción es directamente proporcional al tamaño celular (Berg & Purcell 1977), hecho que ha sido comprobado experimentalmente (Eppley *et al.* 1969; Aksnes & Egge 1991). No obstante, como la superficie celular aumenta más lentamente con el tamaño que el volumen, la demanda celular de fósforo aumenta más que el suministro, si el resto de mecanismos permanecen igual.

A pesar de que los requerimientos celulares de fósforo tienden a ser comparables en organismos de tamaños similares (Lund 1965), la concentración de fósforo es muy variable de un sistema acuático a otro, y en la mayoría de los casos, este nutriente se encuentra en concentraciones subóptimas para el crecimiento de muchas especies (Grover 1991). El efecto de la limitación de fósforo sobre el fitoplancton se ha estudiado utilizando diversas técnicas tales como incubaciones con nutrientes, medida directa de las tasas de absorción de poblaciones, contenido celular de fósforo, cocientes entre nutrientes, producción de exoenzimas para catalizar compuestos de fósforo (Vincent 1981; Smith 1982; Singer *et al.* 1984; Stoddard 1987; Pollingher *et al.* 1988; Elser *et al.* 1990; Pettersson & Blomqvist 1992; Lessin *et al.* 2007; Lin *et al.* 2008), pero ninguna se ha centrado en la comparación del grado de afectación provocado en organismos unicelulares y coloniales, y las posibles estrategias ecológicas para superar esta limitación.

Al igual que ocurre con los nutrientes, la absorción de luz por unidad de clorofila está relacionada con el tamaño celular del fitoplancton (Finkel & Irwin 2001). Sin embargo, las células y colonias más grandes, debido a su mayor densidad de pigmentos por unidad de superficie, sufren un 'efecto de empaquetamiento' que conlleva un uso más ineficiente de los recursos destinados a la absorción de luz. En el caso de las colonias además, esto se ve agravado debido al 'efecto sombra' que las superposición entre sí de las células genera, reduciendo la superficie efectiva para la absorción de luz (Raven 1998).

El tamaño celular también está relacionado con los procesos de 'pérdida' del fitoplancton, principalmente con el hundimiento y la depredación. En relación al primero, Smayda (1970) apuntó que las tasas específicas de hundimiento se incrementaban con el aumento de tamaño celular, por lo que, de nuevo, las células y colonias de mayor tamaño, en principio, tendrían más dificultades para mantenerse en la zona fótica. Para contrarrestar el hundimiento, los organismos fitoplanctónicos han desarrollado diferentes estrategias tales como la formación de espinas, secreción de sustancias de baja densidad, o regulando su flotabilidad con

vacuolas de gas. Algunas de estas adaptaciones suponen un coste energético adicional que eventualmente puede ir en detrimento del crecimiento (Eppley *et al.* 1967; Bienfang 1981; Waite *et al.* 1992). Respecto a la depredación, se ha comprobado que los depredadores zooplanctónicos en numerosas ocasiones se alimentan de presas que tienen tamaños similares o inferiores a los suyos, por lo tanto, las células y colonias de mayor tamaño cuentan con menos depredadores potenciales que las más pequeñas (Woodward *et al.* 2005). Esto afecta a la estructura de tamaños de la poblaciones fitoplanctónicas, haciendo que el tamaño medio de los organismos que la forman tienda a aumentar en condiciones de elevada presión trófica (Porter 1973, 1977; Lynch & Shapiro 1981; Bergquist *et al.* 1985; Knisely & Geller 1986; Vanni 1987). Por lo tanto, el mayor tamaño de las colonias podría conferir ventajas como medida de protección ante depredadores (Boraas *et al.* 1998; Lurling & Van Donk 2000; Mayeli *et al.* 2004; Verschoor *et al.* 2004; Yang *et al.* 2008).

Al igual que pasa con el tamaño, la adopción de una determinada morfología en un organismo fitoplanctónico tiene un efecto directo sobre su ecología (Thompson 1917). La relación entre la forma del fitoplancton y la absorción de nutrientes, las ventajas que ciertas morfologías conferían respecto a otras y el papel ecológico que juega en el ciclo de vida del fitoplancton han sido analizados repetidamente. Margalef (1978) sugirió que la variedad morfológica que exhibían los organismos fitoplanctónicos era una muestra de la respuesta adaptativa al cambiante ambiente en el que viven; idea que para el fitoplancton de aguas continentales sobre todo ha sido desarrollada por Reynolds (2006). La forma de las células y las colonias afecta directamente a la absorción de nutrientes, ya que la superficie expuesta al medio disponible para la el intercambio de sustancias varía dependiendo de la morfología adoptada. En la mayoría de los casos, las colonias reducen la superficie disponible para la absorción de nutrientes; sin embargo, dependiendo de la forma de la misma, la relación superficie-volumen, y como consecuencia, las tasas de asimilación de nutrientes varían sustancialmente. El papel ecológico de la morfología en los organismos fitoplanctónicos también podría estar involucrado en la defensa contra los predadores. De este modo, la adopción de ciertas formas, o la creación de protuberancias y espinas podrían entenderse como la representación de los posibles sistemas de defensa contra posibles ataques (Hessen & Vandonk 1993; Lampert *et al.* 1994; Smetacek 2001).

Objetivos

Debido a su estructura multicelular, los organismos coloniales difieren de los unicelulares, por lo menos, en tamaño y en organización intercelular. Este hecho debe favorecer la existencia de diferencias en los rasgos funcionales básicos entre estos dos tipos de organismos. Teniendo esto en cuenta, el **primer objetivo** de esta tesis fue estudiar las diferencias ecológicas y funcionales entre especies unicelulares y coloniales basándonos en estudios experimentales previos. Para ello, se realizó una exhaustiva revisión de la literatura existente sobre características ecofisiológicas de especies fitoplanctónicas.

La variabilidad en la asimilación de nutrientes entre células del mismo tamaño y en relación a la disponibilidad de nutrientes en el medio es un campo poco estudiado. El tamaño celular determina tanto el flujo de nutrientes que llega a la célula o colonia como sus necesidades nutricionales, por esta razón, es probable que las colonias, que requieren una cierta sincronización entre las células que las componen, hayan evolucionado en un medio que favorezca la reducción de diferencias entre individuos. En relación a esto, el **segundo objetivo** de la tesis fue determinar experimentalmente si las diferencias en absorción de nutrientes entre individuos se ven afectadas por la disponibilidad de nutrientes en el medio, la colonialidad y el tamaño. Para ello, analizamos la variabilidad individual en la absorción de fósforo de dos especies filogenéticamente cercanas: la unicelular *Chlamydomonas reinhardtii* y la colonial *Eudorina elegans*. La microautoradiografía (Brock & Brock 1968; Pedrós-Alió & Newell 1989) proporciona información individual del proceso de absorción del nutriente marcado y permite la monitorización visual del mismo. Se adaptó la técnica para su aplicación en el estudio de la absorción de ^{33}P en fitoplancton.

En relación con la escasez de nutrientes, las posibles estrategias evolutivas del fitoplancton deberían dirigirse al perfeccionamiento de las adaptaciones de estos organismos a determinadas situaciones tróficas, eventuales o continuas. Este es el caso de la producción de fosfatasas extracelulares para hidrolizar compuestos orgánicos de fósforo y aumentar las posibilidades que permitan cubrir los requerimientos nutricionales a ciertos organismos que, bajo determinadas condiciones tróficas, podrían ver limitado su desarrollo (Cembella *et al.* 1984; Jansson *et al.* 1988a). El **tercer objetivo** de esta tesis fue comparar la actividad

fosfatasa entre especies unicelulares y coloniales en diferentes comunidades naturales. Para ello, utilizamos la técnica conocida como ELF (Enzyme Labeled Fluorescence) (Gonzalez Gil *et al.* 1998), que permite la localización directa de la actividad fosfatasa en las inmediaciones donde se produce (Rengefors *et al.* 2001; Nedoma *et al.* 2003; Štrojsová *et al.* 2003). Esta técnica permite visualizar la actividad fosfatásica para cada individuo en comunidades naturales de fitoplancton. Se estudiaron 7 embalses en los meses de primavera y verano, con el objetivo de determinar cuáles son las condiciones que pueden favorecer el desarrollo comparativo de esta estrategia en organismos unicelulares y coloniales.

Aunque la relevancia funcional de las diferentes morfologías que presenta el fitoplancton no está clara, varios estudios han encontrado relaciones directas con la absorción de nutrientes y la defensa frente a los organismos filtradores (Hessen & Vandonk 1993; Lampert *et al.* 1994). En general, la multicelularidad reduce la superficie disponible para el intercambio o toma de sustancias con el medio, pero dependiendo de la forma que presente la colonia, la relación superficie-volumen de la misma puede variar y con ella, la tasa de absorción de nutrientes. Por ello, el **cuarto objetivo** de la tesis fue estudiar el éxito relativo (entendido como la acumulación de biomasa bajo diferentes condiciones tróficas) de diferentes organismos fitoplanctónicos clasificados según su morfología y teniendo en cuenta la colonialidad. Para llevar a cabo este estudio se trabajó con la base de datos de para el estudio del estado ecológico de aguas superficiales del proyecto WISER y datos provenientes de algunos ejercicios de intercalibración para la trasposición de la Directiva Marco del Agua Europea. Los organismos fueron clasificados morfológicamente de acuerdo a su dimensionalidad: unicelulares, filamentos (1D), placas (2D) y globulares (3D). Se estudió si estos tipos morfológicos básicos diferían en su distribución a lo largo del gradiente trófico, si su biomasa se acumulaba bajo determinadas condiciones y si existían patrones consistentes entre los principales linajes filogenéticos.

Métodos

Aproximación metodológica

El estudio de la ecología de las formas coloniales del fitoplancton y sus principales determinantes se aproximó desde dos perspectivas metodológicas: i) a través del análisis de la información ecológica y fisiológica disponible públicamente, con el fin de determinar la posible existencia de patrones generales en estos organismos y ii) a través del diseño y realización de experimentos para contrastar algunas de las hipótesis formuladas. Los resultados de ambas aproximaciones se discutieron dentro del marco general de la evolución y ecología del fitoplancton.

Análisis de datos existentes

Se llevó a cabo un estudio exhaustivo de las limitaciones y ventajas ecológicas conferidas por el tamaño y la forma del fitoplancton a través del análisis de los datos recopilados a partir de dos fuentes: i) las publicaciones de experimentos realizadas con especies aisladas que incluyesen la medida de algún rasgo eco-fisiológico de interés para el estudio (**capítulo 1); y** ii) bases de datos con información sobre la distribución de especies fitoplanctónicas realizadas dentro de programas para la gestión de la calidad del agua (**capítulo 4),** en concreto**,** se utilizó la base de datos de fitoplancton de agua dulce del proyecto WISER y se estudió la distribución de biovolúmenes de diferentes morfologías coloniales y organismos unicelulares a lo largo del gradiente trófico de los lagos definido por el fósforo total.

Métodos experimentales

Existen ciertas técnicas microscópicas que permiten el estudio y seguimiento de respuestas ecológicas individuo a individuo. En el **capítulo 2,** a través del uso de la microautoradiografía y marcaje con el isótopo ^{33}P (Brock & Brock 1968; Pedrós-Alió & Newell 1989), se determinó si los cambios en la disponibilidad de fósforo en el ambiente afectaban a la variabilidad en el proceso de absorción de fósforo a nivel de individuo tanto en una especie colonial como en una unicelular. En el **capítulo 3** se utilizó el marcaje por fluorescencia de la actividad enzimática (ELF) (Gonzalez Gil *et al.* 1998), para investigar los patrones de liberación de exoenzimas fosfatasas

entre especies fitoplanctónicas de comunidades naturales. Esta técnica utiliza un sustrato artificial ELF®97 fosfato (ELFP), que al unirse con la enzima fosfatasa forma un alcohol fluorescente, que precipita en el lugar de unión, y la hace visible bajo un microscopio de epifluorescencia (Huang *et al.* 1992).

Resultados y Discusión

Capítulo 1: caracteres de coloniales *vs.* unicelulares: revisión de datos experimentales

Basándonos en la compilación bibliográfica de resultados experimentales, observamos que, en efecto, la tasa de crecimiento específica guarda una relación negativa con el volumen del organismo fitoplanctónico (unicelular, o colonial). Además, las tasas de crecimiento específicas de organismos unicelulares respecto de las coloniales formadas por células de volumen comparable al de los unicelulares eran significativamente menores. Esto pone de manifiesto que el hecho de configurarse como una unión de células para formar una colonia, implica una limitación para esas células respecto a las libres de un mismo tamaño. Por tanto, existe una limitación para las colonias en el balance de asimilación de nutrientes (proceso que está definido por la superficie total del organismo) y la demanda de los mismos (proceso determinado por su biovolumen celular efectivo) que debe subsanarse evolutivamente de algún modo (p.e., células pequeñas). Teniendo en cuenta los datos referentes a las constantes de saturación media de fosfato, y la relación de este parámetro con la capacidad de asimilación por parte del fitoplancton, no encontramos diferencias significativas que indicasen que una forma estuviera más aventajada que la otra. Aunque la escasez de datos en relación a esta variable podría estar sesgando nuestros resultados, el hecho de que la capacidad de asimilación de fósforo no varíe en relación al tamaño, pero las tasas de crecimiento sí, apuntaría a la imposibilidad de los organismos fitoplanctónicos de evitar las restricciones que el tamaño impone en la difusión y al transporte de nutrientes en las membranas.

Capítulo 2: Variabilidad en la asimilación de P por el fitoplancton con relación al tamaño individual: estudio con automicroradiografía

Gracias a la modificación y aplicación de la técnica de microautoradiografía al proceso de absorción de fósforo pudimos comprobar que, bajo diferentes condiciones tróficas, los individuos de la especie unicelular *Chlamydomonas reinhardtii* cubrían de manera más rápida sus necesidades nutricionales que los de la especie colonial *Eudorina elegans*. Estos resultados refuerzan la idea de que existen limitaciones en la asimilación de nutrientes en los organismos coloniales debido a su mayor tamaño (Aksnes & Egge 1991; Litchman *et al.* 2007a). También se observó cómo a medida que la concentración de fósforo en el medio aumentaba, el efecto del tamaño en el proceso de absorción disminuía (sin llegar a desaparecer). Este hecho podría indicar que, en condiciones eutróficas, el efecto del tamaño es menos notable y las diferencias entre organismos son menores. Este resultado es acorde a los obtenidos en investigaciones anteriores (Litchman *et al.* 2010). De igual modo, cuando la concentración de nutrientes en el medio es escasa, las desigualdades entre organismos unicelulares y coloniales se acentúan. Menor variabilidad entre individuos a concentraciones mayores de nutrientes en el medio indica un mayor potencial para llevar a cabo una división sincronizada de las células, lo cual, evolutivamente, también podría haber favorecido la aparición de las formas coloniales (Doyle & Poore 1974).

Capítulo 3: Comparación de la actividad fosfatasa entre unicelulares y colonias fitoplanctónicas

Los organismos fitoplanctónicos han desarrollado diferentes estrategias para compensar las limitaciones derivadas de la escasez de fósforo en el medio. Entre ellas la producción de exoenzimas capaces de hidrolizar compuestos de fósforo orgánico disuelto. Se monitorizó la actividad fosfatásica de la comunidad fitoplanctónica de 7 embalses en los meses de abril, agosto y septiembre y se encontró que el aumento de la temperatura del agua y el descenso de la cantidad de fósforo total disuelto en el medio favorecían la producción de estas exoenzimas.

De manera interesante, se comprobó que la producción de estas exoenzimas variaba considerablemente entre el fitoplancton unicelular y colonial, siendo los organismos coloniales los que más frecuentemente la presentaban con mucha diferencia. Entre las forma coloniales, las clorofíceas fue el grupo que presentó un mayor número de especies mostrando la actividad. En cianobacterias y diatomeas fue más escasa, aunque al final del período estival *Tabellaria* mostró una elevada actividad. En la discusión se argumenta que las formas coloniales pueden tener ciertas ventajas para el uso de exoenzimas, ya que la actividad de estos queda próxima a distintas células de una misma colonia, particularmente si la actividad se produce en el mucílago, espacios intercelulares o espacios interiores de formas globulares, tal como se observó en muchos casos.

Capítulo 4: Formas coloniales del fitoplancton en lagos de diferente estado trófico

De los resultados obtenidos a partir del estudio de un total de 8527 muestras, provenientes de 1734 lagos que cubrían un gradiente de fósforo de 1 a 1000 µg P L^{-1}, extraídos de la base de datos fitoplanctónicos del proyecto WISER, se observó que las colonias filamentosas alcanzan más biomasa que otras tipos morfológicos a lo largo de todo el gradiente trófico. Dado que no existe ninguna evidencia previa que indique que este tipo morfológico de colonias crezca más rápido que otros (incluyendo los organismos unicelulares), lo más probable es que esta acumulación de biomasa se consiga por la sincronización en su crecimiento o, más probablemente, porque los procesos de pérdida se ven de algún modo aminorados. La misma pauta de mayor biomasa se repite en distintos grupos (p.e., diatomeas y cianobacterias), a pesar de la distancia filogenética existente entre ellos. Lo cual apunta a un mecanismo bastante general. En ambos grupos, hay especies que forman *blooms* bajo determinadas condiciones ambientales. Por ejemplo, para las diatomeas este es el caso de las especies del género *Aulacoseira*, y para las cianobacterias, las del género *Planktothrix* (Takamura *et al.* 1992; Steinberg & Trumpp 1993; Rucker *et al.* 1997; Queimalinos *et al.* 1998; Interlandi *et al.* 1999; Salmaso 2002). No obstante, como el fenómeno se da a lo largo de todo el gradiente trófico, es más probable que la causa de acumulación de biomasa sea la compensación de las pérdidas. De entre las posibilidades existentes, la reducción

del hundimiento y/o de la predación serían los mecanismos más probables (Ferguson *et al.* 1982). Una sedimentación lenta no es una característica propia de las colonias filamentosas, a no ser que posean medio para aumentar su flotabilidad (como las vacuolas de gas en algunas cianobacterias filamentosas) (Walsby *et al.* 1991). Debido a la diversidad de especies que forman la categoría de las colonias filamentosas, parece que la sedimentación no es el principal proceso responsable de la acumulación de biomasa filamentosa, por lo que una reducción en la susceptibilidad a ser engullido o filtrado, podría ser la característica general que hace que los filamentos se acumulen bajo cualquier condición trófica. El zooplancton se alimenta de forma selectiva de fitoplancton de un determinado tamaño (en relación a su propio tamaño), hecho que podría conferir a las colonias, que de manera general son más grandes que las formas unicelulares, una ventaja frente a estas últimas, ya que el rango de potenciales predadores se ve reducido. A partir de los resultados obtenidos parece que únicamente las colonias filamentosas son las que gozan de esta ventaja de manera destacable bajo cualquier situación. Con relación a la distribución de las especies de los distintos morfotipos, se pudo comprobar que las especies unicelulares y filamentosas se distribuyen siguiendo patrones similares a lo largo del gradiente trófico, sugiriendo que no hay unas condiciones particulares que faciliten la diversificación de las formas filamentosas. Por otro lado, observamos que las colonias del tipo placa (2D) y globulares (3D), se distribuían de manera similar a las unicelulares y filamentosas únicamente en condiciones oligotróficas y mesotróficas, mientras que se diversifican más bajo condiciones de eutrofia, especialmente las colonias del tipo placa, que se diversifican notablemente en condiciones hipertróficas. Esto apunta, una vez más, a que las formas coloniales evolucionaron y se diversificaron, probablemente, en ambientes ricos en nutrientes, para después pasar a colonizar ambientes nutricionalmente más empobrecidos.

Igualmente se investigó el rol de las coberturas mucilaginosas en los organismos coloniales ya que su presencia favorece el aumento de tamaño y es una característica común en muchos organismos fitoplanctónicos (especialmente en las colonias); sin embargo no se pudo encontrar una relación clara entre la presencia o no de mucílago y la acumulación de biomasa bajo ninguna de las condiciones tróficas estudiadas.

Conclusiones

1. El tamaño impone una limitación al crecimiento similar en organismos unicelulares y coloniales. Sin embargo, las células que forman parte de una colonia crecen más lentamente que sus equivalentes en tamaño de organismos unicelulares.

2. Una concentración elevada de nutrientes en el medio favorece la sincronización en la división celular, y por lo tanto, favorece el desarrollo de *blooms* y la presencia de organismos coloniales.

3. En condiciones mermadas de fósforo, las formas coloniales presentan más actividad fosfatásica que las formas unicelulares. Se puede argumentar que las formas coloniales pueden sacar provecho de su estructura para aumentar la efectividad de los exoenzimas (p.e., las coberturas mucilaginosas, las cavidades intracoloniales, etc.).

4. Los filamentos acumulan más biomasa a lo largo de todo el gradiente trófico de lagos que cualquier otro tipo de morfología fitoplanctónica, incluyendo las formas unicelulares. Se sugiere que la adopción de esta morfología por algunas especies fitoplanctónicas supone una estrategia especialmente exitosa contra los depredadores.

5. La defensa ante predadores en contraste con la compensación de procesos fisiológicos parece ser la razón más plausible que estaría detrás de la evolución desde formas unicelulares a multicelulares en el fitoplancton. Los ambientes ricos en nutrientes minimizan las restricciones relativas a requerimientos de nutrientes inherentes a la colonialidad. Por ello, sugerimos que probablemente las colonias apareciesen y se diversificasen en ambientes ricos en nutrientes y con fuerte presión de depredación, y a partir de ahí, colonizasen ambientes más empobrecidos en nutrientes.

1. Introduction

Phytoplankters are organisms that exhibit a great diversity in size and morphology, in contrast to the apparent scarce heterogeneity of the environment they inhabit. This fact was early formulated by Hutchinson (1961) as the plankton paradox.

Phytoplankton life-strategies have evolved to overcome the physical and chemical constraints impose by nutrient and light shortage in a turbulent media, and to deal with intra- and interspecific competition for resources and decay processes such as sedimentation, grazing and parasitism. The existing diversity indicates that there is not a unique optimal solution embracing all the situations but many successful options to different combinations of environmental conditions. In this study, the focus will be on colonial life forms and the general ecological constraints that they may face. Since phytoplankton colonies and unicellular forms of different size co-exist at many sites, a main question is determining which can be the advantages for colonies to become a plausible and successful evolutionary alternative to unicellularity.

Table 1. Distribution of unicellulars and colonies in the phytoplankton major taxa. Information (van den Hoek *et al.* 1995).

Division	**Class**	**Unicell**	**Colony**
Haptophyta	Haptophyceae	Majority	Few filaments
Cryptophyta	Cryptophyceae	All	Genus *Bjornberginella*
Dinophyta	Dinophyceae	Majority	Few short filaments
Euglenophyta	Euglenophyceae	All	None
Heterokontophyta	Chrysophyceae	Some	Some
	Synurophyceae	Some	Some
	Sarcinochrysidophyceae	Some	Most
	Xanthophyceae	Some	Some
	Eustigmatophyceae	All	None
	Bacillariophyceae	Some	Some
	Raphidophyceae	All	None
	Dictyochophyceae	All	None
Chlorophyta	Prasinophyceae	All	None
	Chlorophyceae	Some	Some
	Ulvophyceae	Some	Some
	Klebsormidiophyceae	Some	Some
	Zygnematophyceae	Some	Some

Colonial forms are present in many phytoplankton groups, particularly in Cyanobacteria, and Heterokontophyta and Chlorophyta among the eukaryotic groups (Table 1). Colonies are poorly represented in Haptophyceae and Dinophyceae by some filamentous species and, in only the genus *Bjomberginella* is colonial in the Cryptophyceae.

In several classes colonial forms are completely absent: Euglenophyceae, Eustigmatophyceae, Raphidophyceae, Dictyophyceae and Prasinophyceae.

1.1 A first look to the colony dilemma

Small single cells require a lower amount of nutrients to sustain a certain growth rate (doubling time) than larger cells and colonies. However, both large cells and colonial phytoplankton forms have evolved and have been present in nature for billions of years.

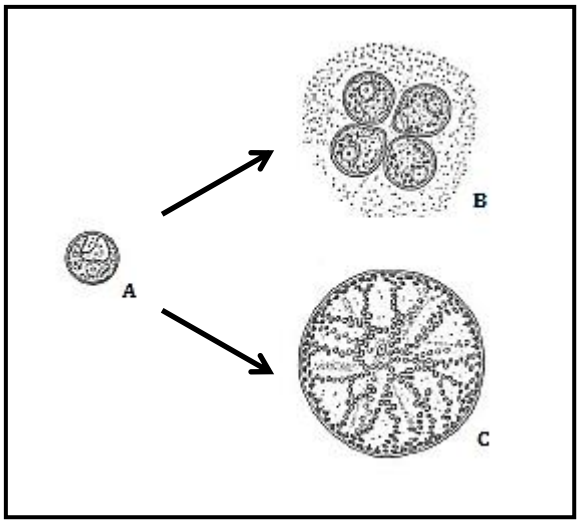


Figure 1. The evolution of a small unicellular organism into a colony or a large unicellular implies different functional constraints and advantages.

Evolving towards a larger single-celled phytoplankter involves higher nutrient cell-quotas but, all other things being equal, the supply do not increase at the same rate. The demand increases proportionally to the cell volume (L^3) and the supply to the cell surface (L^2).

Evolving towards a colony formed by cells similar in size to a unicellular organism, would avoid the problem of a higher nutrient cell-quota for dividing (as the cell volume remains similar), but the problem of a lower nutrient supply will persist, as it is constrained by the colony surface. Therefore, size should confer advantages in some other aspects (Table 2).

Table 2. Potential advantages and disadvantages of colonial organisms relative to single small or large cells (Beardall *et al.* 2009).

Traits	**Advantages of colonies *vs*. single small cells**	**Advantages of colonies *vs* unicellulars of the same biovolume**	**Disadvantages of colonial life forms**
Predation	Avoidance of small grazers	Only one cell from a colony needs to survive predation for subsequent reproduction	More visible than single small cells
	Allelopathic compounds diffuse away rapidly from single cells, whereas diffusion limitation leads to a build-up of chemicals around a colony. Far easier for colony to produce a threshold of allelopathic chemicals	When a predator feeds on a colony, the colony can divide into smaller pieces that may be too small for the predator, and still have capacity for survive	High reward for an specialized predator that obtains a lot of single captures of concentrated food
	Anti-predator advantage of mucilage		
Viral mortality	Avoidance of some viruses and parasites if a pellicle is present		Infection of one cell can result in infection of the entire colony
Resource requirements		More efficient transport of nutrients to cells in a colony compared with a large single cell	Colonies may have greater total resource requirements than single large cells (they may have more

			mitochondria, DNA and mucilage, as well as cell wall) Package effects because of self-shading of chloroplasts
		Net nutrient acquisition and thus growth rates could be better in a colonial form than a single large cell, because of cell surface-to-volume ratios	Limitation by diffusion of inorganic nutrients in colonies is more likely than in isolated cells from those colonies
Specialization	Division of labor/specialization is more efficient and only possible with multiple cells acting together (in the case of multicellular organisms, e.g. *Volvox*)	Division of labor/specialization is more efficient and only possible with multiple cells acting together (multicellular organisms, e.g. *Volvox*)	
Genome replication		Genome replication rate slower for larger cells compared with colonies of small cells More energy and phosphorous needed to replicate genome in a large cell	More copies of genome in a colony compared with a single large cell. It may require a higher level of coordination.

1.2 Size as a key factor in phytoplankton performance

The size of a phytoplankter has certain influence in almost all aspects of its life. Phytoplankton organisms cover a range over nine orders of magnitude, which would be similar to confront the size of a fish to the size of the Manhattan Island (Fig. 2).

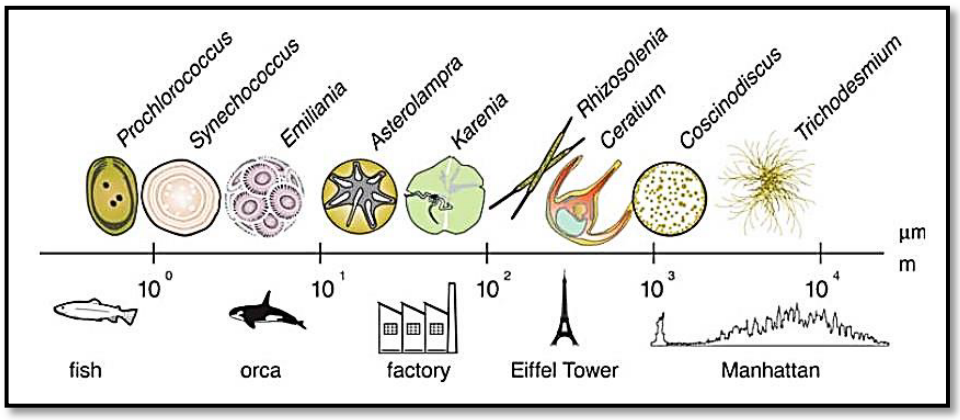


Figure 2. A comparison of the size range (maximum linear dimension) of phytoplankton (Finkel *et al.* 2010)

Perhaps this heterogeneity in size could be expected in such an extremely diverse polyphyletic group as phytoplankton, formed by organisms of unequal evolutionary age, belonging to lineages based in different endosymbiotic events (Falkowski *et al.* 2004). Overall, this range has important consequences as cell size is related, directly or indirectly, to the metabolic activity, growth rate, and numerical abundance (Reynolds 1984; Chisholm 1992), and also affects community structure and dynamics due to the size-dependent interactions (Reynolds 1984). Following, the physiological and ecological consequences of phytoplankton size are briefly reviewed.

1.2.1 Growth rate

The first studies devoted to explore the correlations between cell size and growth rate date back to 1960. Maximum specific growth rates (time $^{-1}$) were found to be negatively correlated to cell volume (Williams 1964; Eppley & Sloan 1966). Later on, it was found that the tendency to growth rate decline was at decelerating rate as cell size increased (Banse 1976; Chisholm 1992). This negative relationship between size and growth rate has been attributed to the size scaling of resource acquisition in phytoplankton (Finkel *et al.* 2004; Irwin *et al.* 2006), because of the more efficient acquisition and use of resources by smaller cells (Raven 1998), or to the scaling properties of intracellular transportation networks (West *et al.* 1999). In summary, the prevalent viewpoint is that large phytoplankton cells are worse

competitors for nutrients than small cells, and hence, are in competitive disadvantage against them in nutrient poor conditions (Smith & Kalff 1982; Grover 1989).

1.2.2 Nutrient availability

To replicate, a phytoplankter organism needs to obtain a minimum quota of inorganic nutrients set by the cell mother demand (Reynolds 2006). Resource availability to phytoplankton cells is a keystone ecological process that shapes communities. Therefore, numerous studies have been carried out using a variety of experimental techniques to investigate how phytoplankton uptake, stored, and consumed nutrients (Turpin 1988; Falkner *et al.* 1989; Grover 1991; Aubriot *et al.* 2000; Hudson *et al.* 2000; Revilla & Weissing 2008; Aubriot *et al.* 2011).

In freshwaters, phosphorus is accepted as one of the most restrictive nutrients for phytoplankton growth (Hudson *et al.* 2000). For that reason, total phytoplankton biomass in freshwater systems is usually approximated as a function of the total phosphorus amount (Oglesby & Schaffner 1978; Stauffer 1991). Also, phytoplankton community composition is related to phosphorus loading in natural systems, as species show contrasting P-affinity, storage capacity and stoichiometric capacity. Nevertheless, although there have been a considerable number of models to relate phytoplankton communities to phosphorus availability (Tilman *et al.* 1981; Tilman *et al.* 1982; Smith & Kalff 1983), such kind of prediction is not very successful due to the necessary oversimplification of the complex auto-ecology of phytoplankton and existing large diversity of species and life forms.

Despite the diversity of life forms or because of it, P-uptake is always fast, even at ambient concentrations of 50 pM of phosphorus or less, microbiological uptake in lake ecosystems is rapid and efficient (Hudson *et al.* 2000). Most phytoplankton organisms can absorb phosphate (P_i) at a faster rate than they use it, and they usually keep this uptake rate until very low P_i concentrations ($<1 \mu g\ P\ L^{-1}$) remain in the water. This luxury uptake may support in some cases two or three generations born in resource deficiency (Reynolds 2006). Therefore, the P concentration in water is the key parameter determining the flux of P towards a cell, since the concentration at the cell surface can be considered as zero, due to this fast uptake.

Following Fick's law, the flux of nutrients towards the organisms can be related to body size by (Pasciak & Gavis 1974; Jumars *et al.* 1993):

$$Q_P = 4 \pi D r_c (C_\infty - C_0)$$

Where Q_P is the flux of nutrients towards a spherical cell, D is the solute's molecular diffusion coefficient, r_c is the cell radius, and $(C_\infty - C_0)$ is the nutrient concentration at the cell surface. Thus, when membrane uptake per unit surface is constant, this rate is proportional to cell surface area (Berg & Purcell 1977). Spherical cells with ratios below 50 μm have a diffusion boundary layer thickness similar to the cell's ratio, which is not affected by the cell's motion relative to the media. Therefore, the equation above can be also applied to cells in motion (Pasciak & Gavis 1974, 1975).

The equation indicates that uptake rates per unit of biomass will be higher in small than in large cells, which has been observed experimentally (Eppley *et al.* 1969; Aksnes & Egge 1991).

Unicellular phytoplankton usually has internal structures separated by minimum distances, which in terms of energy are more affordable than those of larger colonies. As cell size increases, the surface-to-volume ratio declines and the average transport distance within the cell increases, so diffusion becomes increasingly inadequate as a means of maintaining constant solute concentrations throughout the cytosol of the cell (Beardall *et al.* 2009). In that sense, the size of the colonies may star to become critical. However, there are other issues that change with size and become relevant for the final nutrient uptake.

For example, the nutrient environment of small cells most of the times is the same, as their spatial mobility is reduced. On the contrary, large flagellate colonies such as *Volvox*, can undertake 20m of dial movement. As a result, a greater space of resources can be exploited. Large cells can have large storage capacity. Therefore, the simple S/V rationale is not always sufficient, and other trait-offs may have to be taken into account (Bohannan *et al.* 2002). Hence, a question initially faced as "go for being small" or "go for being large" to, respectively, dominate in oligotrophic or eutrophic situations, is open to a wide range of possibilities. As a matter of fact, any strategy by which size can be increased without a proportional increase in the need for the limiting nutrient might create a competitive advantage

(Thingstad *et al.* 2005). Therefore, although some studies and models predict an evolutionary tendency towards small phytoplankton organisms (Raven 1998; Finkel *et al.* 2005; Jiang *et al.* 2005), further considerations indicate there is an evolutionary landscape to explore for large microscopic osmotrophs. Observation shows that large cells and colonies are abundant in natural systems.

Large cells and, particularly, colonies can take more advantage of the production of extracellular enzymes. Extracellular phosphatases have been suggested to play an important role both in species competition for nutrients, and in species succession (Cembella *et al.* 1984; Hino 1988; Jansson *et al.* 1988b; Olsson 1991). They were used as an indication of phosphorus deficiency in phytoplankton populations (Healey & Hendzel 1979, 1980), as cell-specific phosphatase activity has been related to external phosphorus concentration (Dyhrman & Palenik 1999). However, there is an intrinsic problem to release enzymes to the media, and this is that any other species can take advantage of the enzymes activity. Colonial forms, particularly mucilaginous ones, could maintain exoenzymes close to the cells in their external matrix.

1.2.3 Light absorption

Light absorption per unit of chlorophyll is a function of cell size and intracellular pigment concentration (Finkel & Irwin 2001). Larger cells show a larger packaging effect than the small ones (Raven 1998), and colonies, in addition, suffer the 'shading-effect' of some cells superimpose to others, and hence making photon flux less available.

Cells (plasts) with diameters similar to those of wavelengths of photosynthetically active radiation (400-700nm) have imperceptible package effect in photon absorption (Osborne & Raven 1986; Raven 1986). Hence small picophytoplankton cells (cell ratio < 2μm) might show the lowest package effect, and the principal consequence of it would be that, under the same environmental conditions (e.g., photon flux density), cells with the smallest size would show faster production rates than larger cells (Raven 1998). In addition, a lower package effect would lead to a more effective use of resources assigned to light harvesting mechanisms (carbon and nitrogen), and to ATP synthesis, resulting in an energy save that makes smaller cells better competitors than larger cells and colonies for light. All in all, light will rarely be the main determinant of phytoplankton composition as,

eventually, self-shading is inherently related to the success in of the species population growth.

1.2.4 Sinking rate

There is a general agreement between theory and practice that sinking rates increase with particle size (Smayda 1970), and this relationship has been suggested as the explanation for the dominance of small over large non-flagellated phytoplankton stratified systems (Margalef 1978). For a sphere sinking in water, the velocity expected is given by the Stokes equation:

$$v = 2gr^2(\rho' - \rho)/9\eta$$

Where g is the gravitational acceleration (9, 81 m s^{-2}), r is the sphere ratio, ρ' is the cell density, ρ is the water density, and η is the coefficient of dynamic viscosity.

Taking into account this equation, it could be expected an increase in sinking if cells of certain size joint into a colony and their effective size increases. The higher the sinking rate, the shorter the residence time in the photic zone, the shorter the exposition to photon flux, and the less potential for growing. Therefore, size-sinking related issues constitute a relevant selection pressure in the evolution of phytoplankton.

Phytoplanktonic organisms have evolved different strategies to reduce sinking, such as the deviation from the spherical shape, the formation of spines and fibers, the secretion of mucus or the physiological regulation of the cell floatability (Reynolds 2006). Some of the adaptations require energy expenditure, therefore, sinking rates may increase in low-nutrient environmental conditions and under poor light availability (Eppley *et al.* 1967; Bienfang 1981; Waite *et al.* 1992). These are two situations in which small solitary cells are much less constrained to growth than colonies or large unicellulars. When turbulence in the media is high, then the maintenance in suspension is easier for any phytoplankter and other determinants (e.g., nutrient uptake rates) become more relevant for determining the species success (Smayda 1970; Reynolds 2006).

1.2.5 Trophic interactions

There is current evidence that grazing on phytoplankton communities affects its size-structure and often leads to an increase in the average size of phytoplankton assemblage (Porter 1973, 1977; Lynch & Shapiro 1981; Bergquist *et al.* 1985; Knisely & Geller 1986; Vanni 1987). Consequently, a possible advantage for large unicellulars and colonies could be related to the top-down control of the systems by grazers.

A relation between the size of the prey and the size of the predator is expectable, being the size of the organism an upper limit for predation. In other words, smaller predators are more likely to feed on smaller prey, whereas larger predators have a wider range of prey's sizes on which they can feed (Woodward *et al.* 2005). Accordingly, a large volume could help avoiding the smaller predators (i.e., the filter feeding zooplankton species), and in this way, the potential of spanning through larger size is greater for colonial phytoplankton (and multicellular organisms, such as *Volvox*), than for unicellulars.

1.2.6 Functional morphology

There are more species in the lower part of the whole phytoplankton size range. The species-size distribution generally follows a log-normal distribution in which the highest diversity value is at organism size below the median (Vanvalen 1973; Fenchel 1993; Cermeno & Figueiras 2008). However, diversity in morphology is higher at large size. Some part of this morphological diversity probably has a functional meaning.

As with size, the adoption of a certain shape is not a fortuitous process in nature. For the Greeks, the circle and the sphere were considered the perfect geometrical forms, and symbolized the symmetry of the divine. Even in the first attempt to unify the Greek gods into a highest figure (Xenophanes, Colophon c.570-c.475 BC), the spherical shape was attributed to the resulting supreme deity. Since ancient times to nowadays, there has been fascination and theories concerning the spherical shape in nature, from round phytoplankton colonies to spherical-like planets describing spherical-like orbits.

Since the pioneer work ofThompson (1917), the importance of shape and its ecological role has been studied. Lewis (1976) described the relationship between morphology and nutrient uptake, the advantages that a certain shape provided against another, and how this may lead competition between phytoplankton species. A few years later Margalef (1978) suggested that the morphological variability in phytoplankton corresponded to an adaptive response to changing environmental conditions. Reynolds (2006), reinforced the view of phytoplankton shape as a result of a functional adaptive process.

Phytoplankton ecological success or failure, in a particular situation, depends on a fragile balance between gains and losses, which result from the adequacy of functional traits (e.g., related to photosynthesis, resource acquisition, and predation) to the current environmental context. Some authors have emphasized the relevance of gain processes (light, nutrient related traits), and others have pointed to losses as main drivers of phytoplankton temporal succession and, as a consequence, long-term evolution (Smetacek 2001). The phytoplankton morphology can be functionally analyzed mainly under these two views: nutrient acquisition and predation avoidance.

Cell and colony shape directly affects nutrient uptake, as the surface available for absorption varies according to it. In general, coloniality reduces the available surface for nutrient uptake compared to that for the same cells in a free state. However, depending on the morphology, the S/V ratio may differ substantially (Table 3) and, as a consequence, the nutrient assimilation rates.

Regarding predator avoidance, ecological success depends on how efficiently an organism eludes being eaten, filtered, gobbled or infected by their potential grazers, relative to other competitors. In this sense, the role of different phytoplankton cell and colony morphologies reflects the possible defense responses to specific attack systems (Smetacek 2001). Some unicellulars have adopted elongated cell shapes, built spines, protuberances, or horns as morphological defense mechanisms against grazers. Another option is the aggregation of cells to form large colonies that are harder to swallow or filter by zooplankton (Hessen & Vandonk 1993; Lampert *et al.* 1994). Research about this topic has been performed for various phytoplankton and zooplankton species (Boraas *et al.* 1998; Lurling & Van Donk 2000; Mayeli *et al.* 2004; Verschoor *et al.*

2004; Yang *et al.* 2008), showing that formation of colonies is a relative widespread and successful strategy for reducing predation.

Coloniality could also provide protection from viral attack as showed byJacobsen *et al.* (2007) for *Phaeocystis pouchetii.* The rates of infection for unicellulars were higher than for colonies, and even when cells within a colony were infected they induced apoptosis. Nevertheless, very small single-celled organisms can be immune to infection by large viruses because the burst size from the limited resources in a small cell would not be sufficient to maintain the viral population (Raven 2006).

The ecological function of the mucilaginous envelop that some colonies present is not conclusive yet. Their possible roles include extra-storage space for nutrients, density reduction (Lange 1976), defense against parasites, protection against metal toxicity due to its selective permeability (Coesel 1994), and the enhancement of floatability (Walsby & Reynolds 1980; Reynolds 2007). Only few studies have shown some evidence of the possible role of mucilages as nutrient sequestration sites (Cembella *et al.* 1984; Wolf-Gladrow & Riebesell 1997). For predation avoidance, the presence of mucilage causes an increase in size that reduces the possibility of being grazed or filtered by zooplankton, and also, in some species, it prevents from being digested (Hartmann & Kunkel 1991).

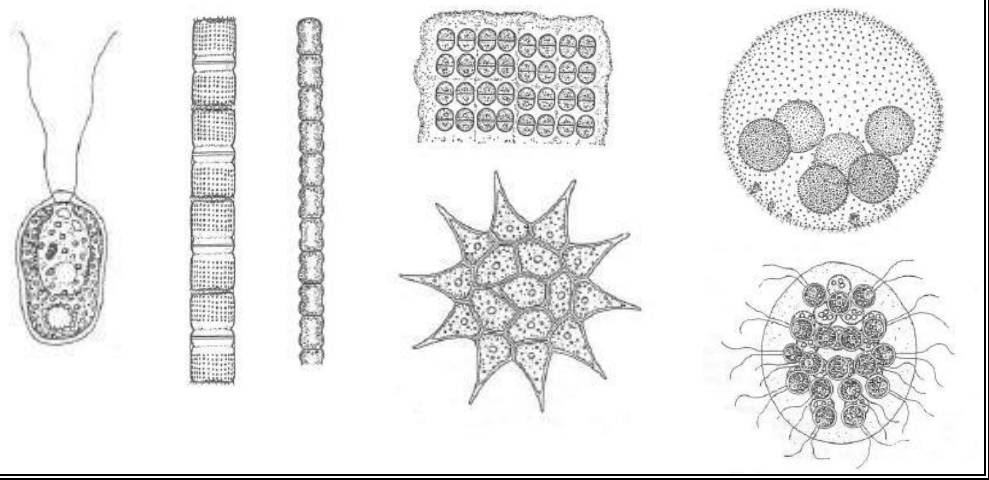
Unicellulars	*Filaments*	*Plates*	*Globular colonies*
Unicellular organism	Colonies built in a one-dimension colony	Cells joint in a two-dimension colony	Spheroids or shapeless colony formations
			

Figure 3. Simple phytoplankton morphological classification

2. Objectives

Colonial organisms differ from phytoplankton unicellulars at least in size and in some intercellular organization simply provided by the colony structure. Therefore, we should expect differences in basic functional traits among the two types of organisms. The **first objective** was to explore trait differences between unicellulars and colonial forms based on experimental studies. A review of the existing literature and experimental studies about relevant ecological traits at species level was approached.

Most colonial forms require certain synchronization of activity among the cells integrating the colony. The nutrient flux arriving to a phytoplankter is a function of its size. The larger the organism the higher the nutrient flux. However, little is known about the variability between cells of the same size, and if the variability in nutrient assimilation varies according to the concentration in the media. Colonies may have evolved better in environments that reduce heterogeneity among the cell responses. Therefore, the **second objective** was to experimentally elucidate whether nutrient uptake heterogeneity among phytoplankters differed across trophic conditions and how this is related to cell size and the colonial form. For these purpose, we analyzed the individual variability of phosphorus uptake in a unicellular (*Chlamydomonas reinhardtii*) and a colonial form (*Eudorina elegans*) using microautoradiography and ^{33}P as tracer. This method provides individualized information on uptake per cell or colony by visualizing the radioactivity incorporated into each cell by the uptake of the labeled substance.

As a strategy to mitigate P limitation, phytoplankton can produce extracellular phosphatases to catalyze the liberation of Pi from dissolved organic phosphorus compounds in the media. The **third objective** was to compare the phosphatase activity between colonial and unicellular phytoplankton species. The approach used was to investigate the extracellular phosphatase release at the level of single-organism using a fluorochrom in several reservoirs during the successional changes of the summer growth period.

Colonial phytoplankton displays an amazing variety of shapes. The functional relevance of all of them is not obvious. The basic morphological classification is distinguishing between unicellulars and colonial organisms, and among the latter according dimensionality (1D to 3D), that is, filaments, plates and globular colonies

The **fourth objective** was to study the relative success of these different phytoplankton morphologies in attaining population biovolume at different ecosystem trophic states taking advantage of a large dataset prepared based on the WISER project data and some EU Water Framework Directive intercalibration exercises.

3. Materials & Methods

3.1 Methodological approach

The exploration of the main determinants of the ecology of colonial phytoplankton was performed following two complementary methodological approaches: i) analyses of existing data generated for other purposes to unveil general patterns and ii) experimental designs to address some specific questions. The results from each approach are discussed in a general framework of phytoplankton ecology and evolution.

3.1.1 Existing data collation and analysis

An exhaustive study of limitations and advantages imposed by size and shape in phytoplankton ecology was carried out, by analyzing existing data from two sources: i) publications on experiments with isolated species that included the measurement of some ecophysiological traits and ii) databases of phytoplankton species distribution elaborated for water quality purposes.

3.1.1.1 Bibliography survey

During the last decades, a vast number of studies have addressed the physiological and ecological performances of phytoplankton organisms at different levels. However, most of these studies focused on -bulk community responses rather than on single-species. Generally, the specific traits of the species were derived later by statistical interpretation, without the capacity of being precise about individual species unless they were extremely dominant in the community. In part, this was due to the lack of techniques able to measure individual response in field studies. The alternative was the experimental determination of some traits, yet these necessarily were limited to a few species in each experiment. Through years, a wealth of information have been accumulated that combined could provide clues about the eco-evolutionary history of phytoplankton. As many of the species studied were colonial organism, these studies are a source of information to compare traits between unicellulars and colonial forms (objective 1). In **chapter 1** we include the results of the data analysis resulting from the information gathered in an extensive bibliographic survey on phytoplankton ecophysiological experiments.

3.1.1.2 Phytoplankton distribution databases

For a decade, since the EU Water Framework Directive (2000/60/EC) (WFD) came into force, the development of indices and tools to assess and monitor the ecological status of freshwater environments is subject to numerous research and monitoring activities through Europe. The WFD has stimulated the development and improvement of a large array of different phytoplankton parameters, including biomass metrics, such as chlorophyll, cell numbers and biovolume, taxonomic composition metrics, such as proportions of cyanobacteria or other indicator taxa. The applications of many of the metrics developed requires of species lists and biovolume evaluation, which have become available for a large number of water bodies throughout Europe. Initiative for the intercalibration of metrics, and European projects for methodological improvements (e.g. WISER project) have facilitated the development of large databases from which other type of studies can be derived. The biovolume achieved by distinct life forms across trophic gradients can be an indication of their relative success and living constraints (Objective 4). In **chapter 4** we use the WISER freshwater phytoplankton dataset to explore the biovolume distribution of basic colonial forms across the trophic gradient as indicated by the total phosphorus (TP) in the system.

3.1.2 Experimental procedures

Current microscopic techniques allow approaching the physiological performance of phytoplankton individuals. Therefore, in addition to the traditional bulk activity of the community, the response of each species (in mixed populations) can be determined and, furthermore, the intra-specific variability can be determined as well. Two different techniques were applied: microautoradiography, to explore how individual variability in P-uptake changes across trophic gradients (objective 3), and enzyme labeled fluorescence (ELF), for the investigation of patterns of phosphatase release among phytoplankton species (objective 4).

3.1.2.1 Microautoradiography

Microautoradiography (Brock & Brock 1968; Pedrós-Alió & Newell 1989) provides individualized information on physiological activities of processes that can be

labeled with a radioactive isotope. The current availability of a P isotope of short life (^{33}P) has renewed the interest for the microautoradiography technique applied to P-uptake. We set up an experimental design to study how the intra-specific variability in P-uptake changes across a P-gradient covering most of the range found in nature. In **chapter 2**, we show the results of adapting and improving the microautoradiography protocols for the analysis of P-uptake in two chlorophyte species, *Chlamydomonas reinhardtii* (unicellular) and *Eudorina elegans* (colonial form). We specifically explore how individual variability relates to size.

3.1.2.2 ELF-technique

Extracellular phosphatases catalyze the liberation of phosphate (P_i) from organic phosphorus compounds. It represents a physiological strategy to try to achieve the necessary minimum nutrient cell quotas for duplication. The use of an enzyme labeled fluorescence method (ELF) (Gonzalez Gil *et al.* 1998) allows direct localization of phosphatase activity in the cell immediate surroundings (Rengefors *et al.* 2001; Nedoma *et al.* 2003; Rengefors *et al.* 2003; Štrojsová *et al.* 2003; Cao *et al.* 2005; Štrojsová & Vrba 2006; Litchman & Nguyen 2008; Štrojsová *et al.* 2008). This technique uses an artificial substrate called ELF®97 phosphate (ELFP) that once cleaved by external phosphatases, forms a fluorescent alcohol precipitate (Huang *et al.* 1992), which is visible under an eplifluorescence microscopy. In **chapter 3**, we applied the technique to samples from three seasonal samplings of phytoplankton from several river Duero reservoirs in order to investigate whether colonial forms shows more phosphatase activity than unicellulars and how these changes throughout the successional summer sequence.

3.2 Numerical methods

A set of statistical methods are used throughout the result chapters (Table 3). All numerical analysis were performed with the statistical software S-PLUS 6.1 (TIBCO Software Inc.), with the exception of quantile regression in chapter 4, which was performed with R 'quantreg' package (Cade & Noon, 2003; R Development Core Team, 2007; Koenker, 2009).

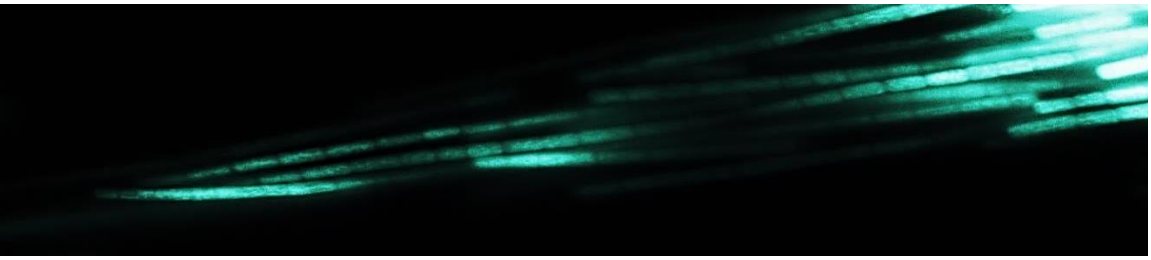
Table 3. Numerical methods applied.

Method	Application example	Chapter
Kruskal-Wallis one-way analysis of variance (non-parametric)	Testing whether unicellular and colonial cell size have a similar distribution. Data non-normally distributed.	1
Wilcoxon-test of mean (non-parametric)	Comparing size sets of measurements to assess whether their population mean differs. Data non-normally distributed.	1
Student's *t*-test	Testing if two sets of a certain eco-physiological traits for unicellulars and colonies are significantly different from each other. Normality assumed.	1
Simple linear regression	Modeling the relationship between P-uptake rates and phytoplankton size.	1,2
Potthoff analysis for the comparison of regression coefficients	Determining whether the linear relationships between P-uptake rate and size differ across levels of P concentration.	2
Principal Component Analysis (PCA)	Summarizing the main patterns of environmental change across the river Duero reservoirs.	3
Discriminant Analysis (DA)	Create an equation that minimizes the possibility of misclassifying cases of alkaline phosphatase activity (APA) according to temperature and total dissolved phosphorus in the media	3
One-way ANOVA	Testing whether the distributions of average species biovolume differ among life-forms and across trophic states.	4
Generalized Linear Models (GLM)	Relating average species biovolume to total phosphorus and life-form categories.	4
Quantile regression	Relating phytoplankton cell size and total phosphorous concentration.	4

4. Results& Discussion

4.1

Chapter 1: Colonial *vs* unicellular phytoplankton traits: a review of experimental data



"All space is relative. There is no such thing as size. The telescope and the microscope have produced a deadly leveling of great and small, far and near.

The only little thing is sin; the only great thing is fear!"

(David H. Keller)

4.1.1 Introduction

Traits determined experimentally can be used for understanding population dynamics and community structures across environmental gradients under the assumption that they reflect the eco-evolutionary history behind the species. Here, we aim to compare unicellular and colonial life forms through a literature survey of experimental studies that evaluated relevant ecological traits, such as maximum growth rates, half-saturation constants for phosphorus uptake, and sinking rates.

4.1.1.1 Bibliographic compilation

Data from 134 published experimental studies were collated. Together they included data for 87 unicellular and 107 colonial species of phytoplankton (both marine and freshwater). A list of the studied species and the traits compiled is included in Annex I. The ecological traits with information for many species included the phosphate uptake half-saturation constant (PO_4^{3-} K_m), maximum growth rate (G_r) and sinking rate (S_r). Other information compiled (e.g., photosynthetically optimal light intensity (I_{opt}), maximal production rate (P_{max}), nitrate and ammonium uptake half-saturation constants (NO_3^- K_m, NH_4^+ K_m) did not included enough species to be statistically useful. In the experiments, growth rate was measured under different experimental temperatures. Thus, to perform comparisons, we normalized the growth rate to a temperature of 20^{o}C, for which we used a Q_{10} value of 1.64 (Sal & Lopez Urrutia 2011). If cell volume was not stated in the respective publication, it was calculated using a geometric approximation to the organism shape, and mean size values from the literature (Annex II).

4.1.2.2 Data analyses

The relationships between nutrient-related traits and cell volume are typically linear when both variables are log-transformed (Litchman *et al.* 2007b); consequently, we performed all analyses on $\log_{10}$-transformed values.

To visualize size-related variability, we classified the taxa into size categories (namely, $<10^2$, 10^2-$<10^3$, 10^3-$<10^4$, 10^4-$<10^5$, and $\geq 10^5$ μm^3). For colonies, we distinguished between cell size and the size of the whole colony. We use the term "organism size", to refer either to the unicellular or the whole colony size. *Kruskal-*

Wallis test was performed to check the differences in trait mean values among size categories between both life forms. If significant differences were found, then pairwise comparisons were performed using a *Wilcoxon*-test.

In order to consider potential phylogenetic differences, we considered the taxonomic groups: Cyanobacteria, Chlorophyta, Bacillariophyta, Chrysophyta, Cryptophyta and Xanthophyta for which there were a sufficient number of colonial and unicellular representatives (Table 4.1.1).

Table 4.1.1. Number of species compiled for growth and sinking rate (Gr20, Sr), phosphate uptake half-saturation constant (PO_4^{3-} K_m), for the unicellular (U) and colonial forms of Cyanobacteria (Cya), Chlorophyta (Chlor), Bacillariophyta (Diat), Chrysophyta (Chry), Cryptophyta (Cry), Xanthophyta (Xan).

	Cyan		Chlor		Diat		Chry		Cry		Xan		**Total**
	U	C	U	C	U	C	U	C	U	C	U	C	
Gr_{20}	3	24	12	16	22	15	2	3	9		1	3	110
PO_4^{3-} K_m	1	9	5	5	11	6			1				38
Sr	1	9	8	5	14	16	1		4			1	59

4.1.2 Results

4.1.2.1 Growth rate

Growth rate tends to decrease with unit volume (Fig.4.1.1), both considering the original data and normalized to 20ºC.Original growth rate variability shrinks when normalized to consider temperature differences. However, the relationship splits into two divergent lines.

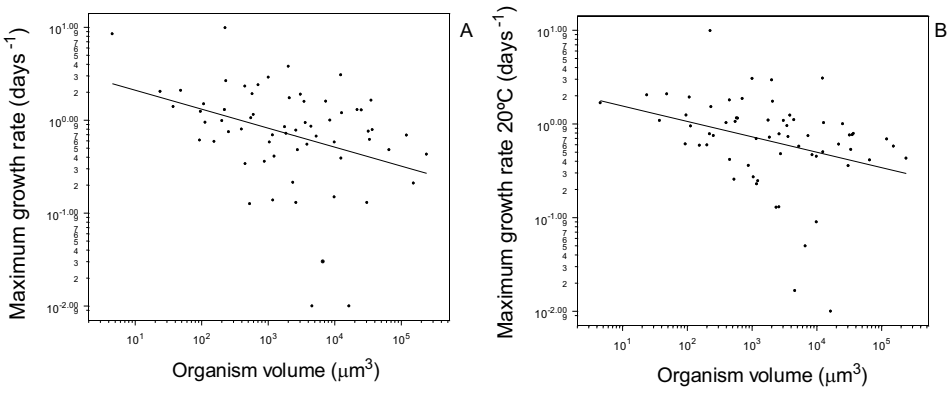


Figure 4.1.1. Relationship between growth rate and organism volume. Note the logarithmic scale in the axes. Lines plotted are from least-squares regression in Table 4.1.2

Table4.1.2. Least-squares regressions of the original-logarithmic and normalized-logarithmic growth rate values of Gr, Gr20respectively*vs*organismvolume (Fig. 4.1.1).

	Intercept	**Slope**	**Slope p-value**	**R -Squared**
G_r	0.5277	-0.2044	0.0003	0.1326
G_{r20}	0.3560	-0.1645	0.0080	0.1096

Both, unicellular and colonial organisms show a declining trend in growth rate with increasing organism volume (Fig. 4.1.2). Unicellular organisms show apparently higher G_{r20} than colonial species (mean 1.12 vs. 0.71 day $^{-1}$, respectively; *t*-test *p*-value 0.01). However, when we consider the size class distributions (Fig. 4.1.2) there are no significant differences between the two life forms within the classes (Kruskal-Wallis *p*-value 0.07). Therefore, since there were no colonial forms below 10^2 µm^3 and above 10^5 µm^{3}organism volume, the apparent discrepancy between the mean growth values results from differences in the size distribution of the two life forms.

Interestingly, when comparing growth rates classifying the colonies according to their cell size rather than the whole organism size, differences emerge (Kruskal-Wallis *p*-value 0.015). Colonial growth rates are systematically lower than those of the unicellular species of the same cell size class (Fig. 4.1.2B), although only for the class <10^2 µm^3 the difference is statistically significant (*t*-test *p*-value 0.0046).

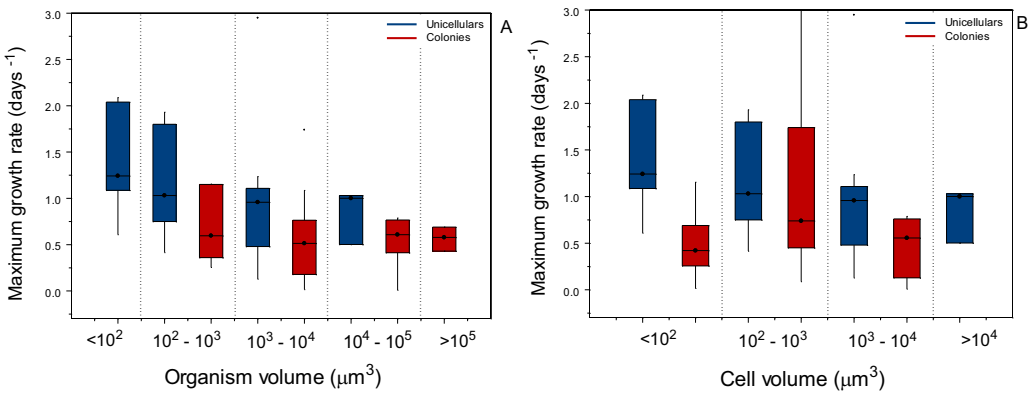


Figure 4.1.2. Growth rate *vs* organism size (A), and cell size (B) classes in unicellular and colonial species.

No significant differences were found between the main taxonomic groups (Fig. 4.1.3) for which sufficient data was available (Kruskal-Wallis *p*-value 0.04). Within groups, growth rates for unicellulars and colonial forms weren't different neither in diatoms (colonies Gr_{20} 1.20 day^{-1}, unicellulars 1.027 day^{-1}, *t*-test *p*-value 0.49) nor in chlorophytes (0.66 *vs* 1.11 day^{-1}, respectively, *t*-test *p*-value 0.07). In contrast, cyanobacteria showed a large difference in growth rate between colonies and unicellular forms (0.91 *vs* 34 day^{-1}, respectively; *t*-test *p*-value 0.0007); the high growth rates achieved by colonial cyanobacteria are due to the thermophile species *Synechococcus* sp., and *Synechococcus lividus,* that were originally cultured at 45 and 52 ºC. Cell volume did not show significant differences between unicellulars and colonies in any taxonomic group (Fig. 4.1.3 B).

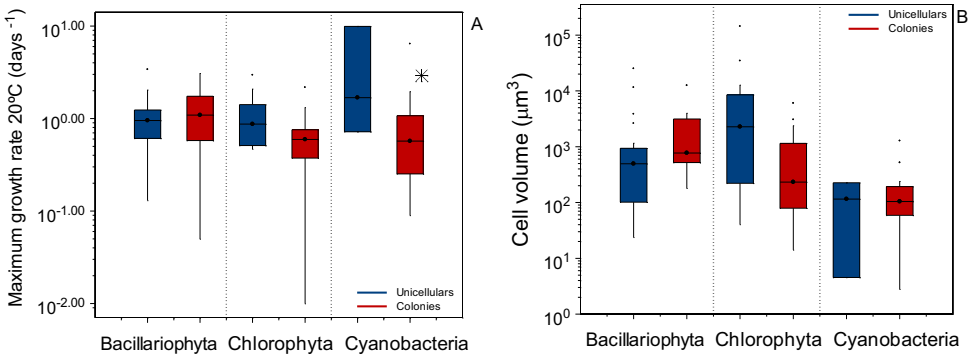


Figure 4.1.3. Maximum growth rates in different taxonomic group (A). Cell volume *vs* taxonomic group (B).

4.1.2.2 Phosphate uptake half-saturation constant

Compared to data for growth rates, phosphate half-saturation constant values (PO_4^{3-}-K_m) were scarcer in the literature revised (Table 4.1.1). PO_4^{3-}-K_m data for unicellulars with volumes >10^3 µm^3, and colonies with volumes >10^5µm^3 were rare (Fig. 4.1.4 A). There were no difference in average PO_4^{3-}-K_m values between colonial (5.25 µmol L^{-1}), and unicellular (7.70 µmol L^{-1}) organisms (*t*-test *p*-value 0.71); neither there were differences when considering the organism volume classes (*Kruskal-Wallis p*-value 0.59).

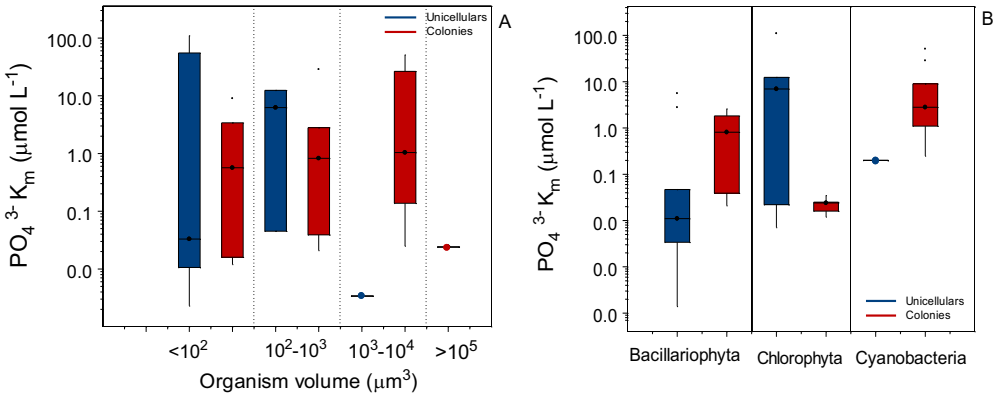


Figure 4.1.4. Phosphate uptake half-saturation constant considering organism size and taxonomic groups. Note the logarithmic scale.

PO_4^{3-}-K_m differed between diatoms, chlorophytes, and cyanobacteria (average values 0.86, 13, and 9.89 µmol L^{-1}, respectively) (Fig.4.1.4 B) (*Kruskal-Wallis p*-value= 0.014). However, no differences between unicellular and colonial organisms were found within each group (*Wilcoxon* test *p*-values 0.09, 0.3, 0.2, respectively for Diatoms, Chlorophytes and Cyanobacteria).

4.1.2.3 Sinking rate

A general main tendency of increasing sinking rates with increasing organism volumes can be observed (Fig.4.1.5). However, no significant differences between colonial and unicellular mean sinking rates or between taxonomic groups exist. Nevertheless, the statistical analysis can be highly conditioned by the scarcity of data (Table 4.1.1).

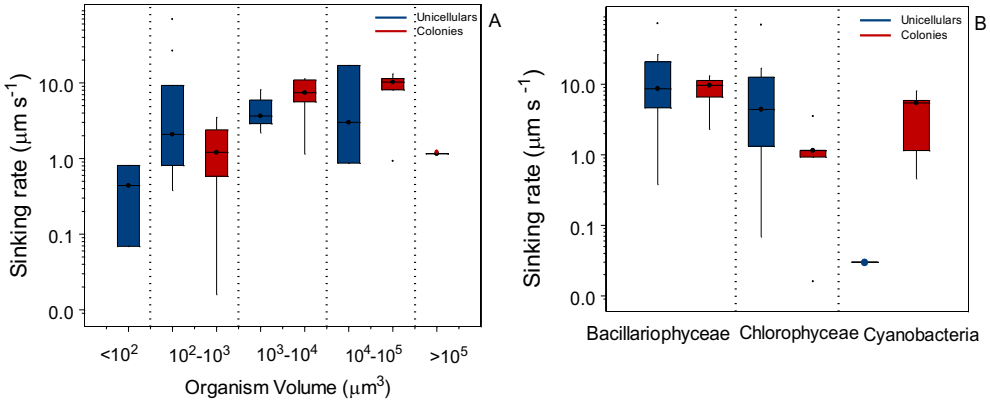


Figure 4.1.5. Sinking rate *vs* organism volume and taxonomic groups.

4.1.3 Discussion

The compilation of maximum specific growth rates showed a negative correlation between them and biovolume in phytoplankton organisms (Fig.4.1.1), indicating a faster replication of the small organisms. These results are in agreement with previous studies (Fenchel 1974; Banse 1976; Reynolds 1984; Irwin *et al.* 2006; Finkel *et al.* 2010; Edwards *et al.* 2012), and in accordance with the scaling of resource

requirements for growing with cell size (Reynolds 2006). In large organisms, intracellular transport distances increase, affecting negatively to resource diffusion and slowing down growth rates (Beardall *et al.* 2009).

Simply because unicellular organisms tend to be smaller than colonial ones, their average specific growth rate is faster (Fig. 4.1.2). However, when the comparison is made for a range of similar size, differences are no longer significant, indicating a general constraint of size upon specific growth rates irrespective if they are unicellular organisms or colonies. Interestingly, when comparisons are made based on cell size rather than the whole colony size, then differences between unicellulars and colonial forms turn to be significant. Specific growth rates are smaller for colonial organisms than for unicellulars of the same size, particularly for organisms with the smallest cells ($<10^2$ μm^3). Despite the large variability, this fact indicates a physical constraint for colonial phytoplankton related to the balance between the arrival of resources determined by the whole organism surface and the consumption (use) of these resources related to the effective cell biovolume, which eventually determines growth rate. Since transport within colonies is less organized that within cells, in order to achieve a similar specific growth rate than unicellulars of the same size, a colonial organism has to have a smaller effective biovolume (that is smaller cells). Thus, their cells will show smaller growth rates than the corresponding free cells of the same size. The fact that we did not find significant differences between the main taxonomic groups (Fig. 4.1.3) suggests that the physical constraints are fundamental, and override any potential adaptive issue.

Regarding phosphorus uptake half-saturation constant, we did not find any significant difference between single cells and colonies nor among size classes. However, there were marked differences among the main taxonomic groups (Fig. 4.1.4B). Because the capacity to assimilate phosphorus does not change with size, it means that phytoplankton organisms cannot avoid the restrictions imposed by size-related diffusion and transport constraints by enhancing the assimilation capacity. Therefore, colonies and unicellulars would tend to grow in nutrient–rich environments to achieve similar growth rates than smaller organisms. An increase in the concentration of the limiting nutrient (e.g., phosphorus), would result in an increase of the maximum cell size present.PO_4^{3-}-K_m differences among the main taxonomic groups agree with their common preferences for environments of

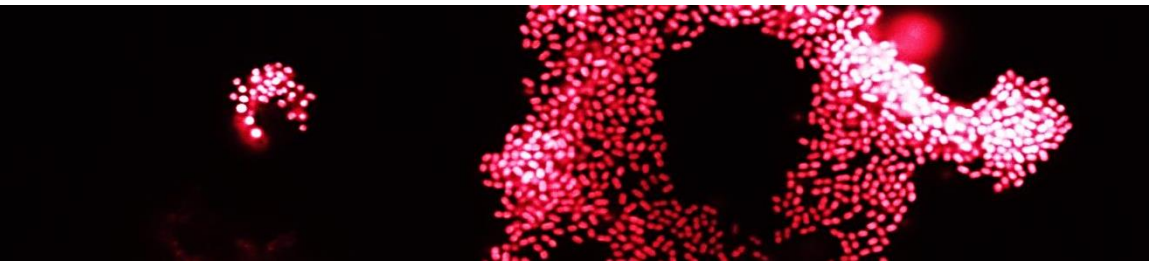
certain trophic degree, as we will see later in another chapter. Although the three groups considered tend to grow in nutrient rich environments, PO_4^{3-}-K_m increases from diatoms to Chlorophyta and Cyanobacteria, which is consistent with the rank of trophic conditions in which they are dominant.

The compiled data indicate that sinking rate increases with increasing volumes, as expected from purely physical restrictions (i.e., Stokes Law) (Smayda 1970; Padisak *et al.* 2003). However, the sinking rates were no significantly different between unicellular and colonial phytoplanktonic organisms, neither between unicellulars and colonies of similar volumes, nor between taxonomic groups. Nevertheless, the result could be biased by the scarce experimental information available about this trait, and the large variability observed. Beyond statistics, diatoms were the ones showing the highest sinking rate (Fig.4.1.5), which is consistent with their silica valves and, consequent, higher density per unit of volume.

In summary, from this review of experimental data on eco-physiological traits, there is no evidence that colonial forms have an obvious advantage over unicellulars in any situation. They simply follow the same size constraints than unicellulars and, therefore, they share the requirements of very large cells. On the other hand, colonial forms are broadly represented in some groups and in certain environments become dominant. Which are their ecological advantages over the unicellulars in those contexts? One option relies in a loss factor not considered here: grazing. There are studies suggesting that the limitation of picophytoplankton biomass in high-nutrient environments might be due to control by grazers (Fogg 1986; Happeywood & Lund 1994; Irigoien *et al.* 2005). Zooplankton consumption rate depends on both zooplankton and phytoplankton size. In general, zooplankton grazers are larger than their phytoplankton prey, and large grazers can potentially feed on a wider size range of phytoplankton organisms than small ones (Reynolds 1984). More importantly, zooplankton grazers do not just eat indiscriminately but selectively feed on phytoplankton cells that are of certain size (relative to their own size), presumably, as a way to maximize energy gains. This could give colonies and large single cells an advantage, as they have fewer potential predators than small unicellulars. Some authors have suggested an evolution towards extremely small sizes in the absence of grazers (Jiang *et al.* 2005).

4.2

Chapter 2: Size-related P-uptake variability in phytoplankton: a microautoradiography approach



"We have a method, and that method helps us to reach not absolute truth, only asymptotic approaches to the truth — never there, just closer and closer, always finding vast new oceans of undiscovered possibilities."

(Carl Sagan)

4.2.1 Introduction

Phosphorus uptake has been traditionally estimated as the mean response of mixed phytoplankton populations in nature or as a cell average response of cultures. Therefore, little is known about the differences between cells of a species population. Here, we study the individual variability of phosphorus uptake under different concentrations in the media for a unicellular (*Chlamydomonas reinhardtii*), and a colonial organism (*Eudorina elegans*), which are phylogentically close. We aimed, to study how individual variability was affected by the individual size and if this variability changed under different concentrations in the media. More explicitly, we aimed to evaluate whether size constraints to nutrient uptake relax at increasing nutrient concentrations in the media. The hypothesis is that higher total nutrient uptake by a population when switching from low to high nutrient concentrations can occur because each individual increases its uptake proportionally, but differences among them remain, or because differences between individuals decrease. As cell nutrient requirements are finite, one may expect that the latter option is more reliable than the maintenance of the variance among individuals. According to this hypothesis, higher growth rates will be maintained in nutrient rich conditions because a larger proportion of cells would achieve the nutrient cellular quota to divide in a certain time than at lower concentrations in the media. This hypothesis was tested using a microautoradiography approach (Brock & Brock 1968; Pedrós-Alió & Newell 1989), which provides individualized information on uptake per cell or colony by visualizing the radioactivity incorporated into individual cells by the uptake of a labeled substance. For our purpose of studying P-uptake, we adapted a microautoradiography protocol to the use of ^{33}P.

4.2.1.1 Species cultures

The Chlorophyceae species *Eudorina elegans* (SAG29.87) and *Chlamydomonas reinhardtii* (SAG11-32a) were provided by the SAG (Sammlung von Algenkulturen Göttingen) culture collection of algae. Cultures used for inoculation during the experiment were at the exponential phase. The growth media for both species was the enriched Desmidiacean medium $MiEB_{12}$ (http://sagdb.uni-

goettingen.de/culture_media/07%20Desmidiacean%20Medium.pdf). All stocks and experimental cultures were conducted at 20±1ºC in a 12:12h (L:D) cycle, under controlled light conditions. In order to minimize any bias due to the incubation setting, the flasks were shaken manually once a week and rearranged randomly.

4.2.1.2 Experimental design

The experimental procedure is summarized in Fig.4.2.1.

Phosphate incubation gradient

In order to study the phosphate uptake at individual level, a set of five increasing phosphate concentrations was selected for incubations (Table 4.2.1), with the aim to replicate the main trophic states usually recognized in freshwaters (Wetzel 2001).

Table 4.2.1. Selected concentrations for experimental settings

Reference trophic state	$[PO_4^{3-}]$ µM
Ultra-oligotrophic	0.195
Oligotrophic	0.395
Mesotrophic	0.795
Eutrophic	1.595
Hyper-eutrophic	3.195

Phytoplankton cultures were cultivated with $MiEB_{12}$ growth medium with low phosphate (0.1 µM) during the month previous to the experiment, in order to have organisms under phosphate-stress.

Incubation time

We estimated the time for incubations based on the P required for a cell to double its content, and the P flux determined by diffusion. P required was estimated as 0.18 mol PL^{-1} based on an amount of carbon per cell volume of 18.8 mol C L^{-1} (Reynolds 2006), and C:P stoichiometry of 106:1.

The P diffusion flux into a spherical cell (Q_P), depends on the nutrient diffusion coefficient (D), the cell radius (r_c) and the concentration gradient between the cell surface and the ambient water (Jumars *et al.* 1993):

$$Q_P = 4 \pi D r_c (C_\infty - C_0)$$

In the case of phosphorus, the constant P is equal to 21×10^{-9} m^2 s^{-1}; C_0 is assumed to be 0 at the cell surface, and C_∞ is the concentration of P in the media selected above.

According to these assumptions, we estimated that a time series of 4, 8, 16, 32, 64 and 128 minutes, will be suitable to estimate individual P uptake rates under the different P concentrations selected and organisms considered. The goal was to assure that, within this temporal range, there will be sometime steps suitable for the estimation of individual uptake rates, that is with radioactive labeling not to low (undifferentiated from background) and not too high (radioactive labeling saturating the individuals autoradiography).

$^{33}PO_4^{3-}$-additions

We used $^{33}PO_4^{3-}$ tracer from Perking Elmer (100µCi~3700 kBq, specific activity 5141.4 $Cimmol^{-1}$, Perking Elmer). Considering the experimental gradient (Table 4.2.1), a total of five working solutions (WS) were specifically prepared to achieve a $^{33}PO_4^{3-}$:PO_4^{3-} ratio of 1:10280 in all the experiments (Table 4.2.2).

Table 4.2.2. ^{33}P-labelled working solutions (WS) for the experiments (Fig.4.2.1)

Reference trophic state	**$^{33}PO_4^{3-}$-activity (µCimL^{-1})**	**[PO_4^{3-}] (µ)**
Ultra-oligotrophic	2.0005	2
Oligotrophic	4.0011	6
Mesotrophic	8.0021	14
Eutrophic	16.004	30
Hyper-eutrophic	32.008	62

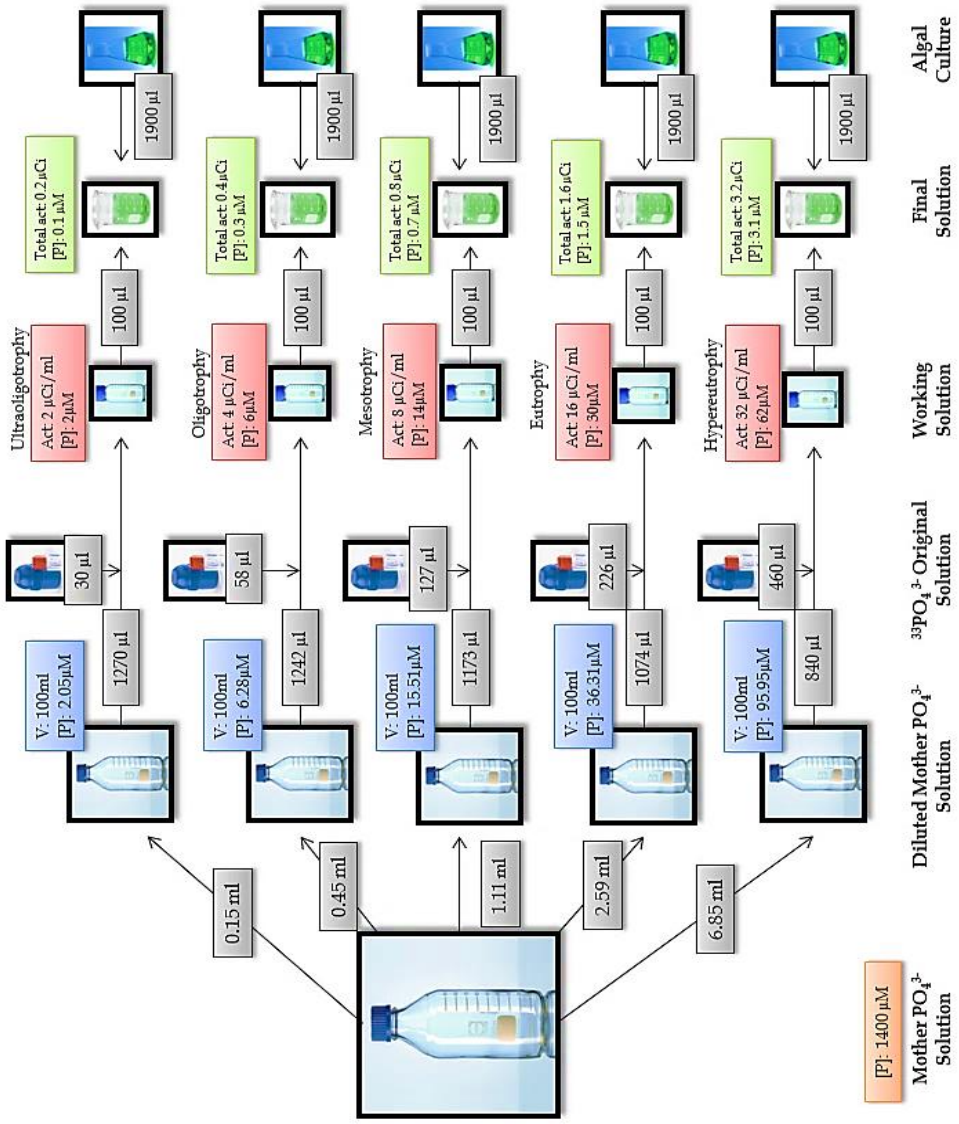


Figure 4.2.1. Experiment scheme

The two phytoplankton species, *Eudorina elegans* and *Chlamydomonas reinhardtii,* were incubated at the same time, six wells for each species (Fig. 4.2.2)

Figure 4.2.2. Experimental 12-well manifold

The final volume set for incubations was 2mL. Aliquots of 1900 µL from the algal cultures, and aliquots of 100 µL from the corresponding working solution (Table 4.2.2) were incubated at 20ºC during 4, 8,16,32,64 and128 minutes, respectively. Labeling was stopped by infinitely diluting the $^{33}PO_4^{3-}$with non-labeled phosphate solution (1.4 mM).

Microautoradiography

We modified the protocol described in Meyer Reil (1978), in order to adapt it to$^{33}PO_4^{3-}$uptake in phytoplankton. Microautoradiograms were prepared by filtering (at low pressure) the incubation volume through two 0.2µmpore-size polycarbonate filters (Isopore, Millipore). Filters were left drying in a cardboard box provided with a bed of absorbent paper for 30 minutes, and stored at -20ºC until processed. In the dark room, slides were dipped for 5 seconds in NTB-2 (Kodak) mixed with 1:1 0.2% low-melting point agarose (Štrojsová *et al.* 2010), which previously had been heated at 43ºC in a warm water-bath during 45 minutes. Slides were dried and placed for 5 minutes on a previously frozen metal piece. Filters were carefully placed face-down on the emulsion-covered surface of slides. The slides were dried and exposed in the dark at 3ºC for 3 days. The microautoradiograms were developed for 2 minutes in Kodak Dektol developer

(diluted 1:3 with distilled water), followed by a 30 seconds stop rinse in Milli-Q water, and a 4 minutes soak in Kodak Fixer. Slides were immerse in glycerol (1%) for 2 minutes, and stored in a desiccator inside a box protected from light for 6 hours. Filters were gently peeled off, and cells in the emulsion were stained with a drop of Citifluor mounting solution, and covered with a coverslip.

4.2.1.3 Image acquisition, processing and P-uptake calculation

Microautoradiography (MAR) images were taken at randomly selected filter positions, using an eplifluorescence microscope Zeiss Axio Imager, with a 120 X-cite lamp, and a coupled camera AxioCamMrm with the acquisition software Axio Vision for PC, under simultaneous epifluorescence and light-transmitted illumination, at 63x magnification. For each incubation time, based on the abundance of each species, an average of 40 MAR-images for *C. reinhardtii*, and 10 for *E. elegans* were captured. Image processing was performed using *ImageJ* image analysis software (Rasband 1997). The total number of cells and colonies processed was 1398 and 320 respectively (Table 4.2.3). Some examples of the P-uptake visualization are included (Fig. 4.2.3-Fig.4.2.7).

Table 4.2.3. Number of *C. reinhardtii* cells and *E. elegans* colonies processed.

Reference trophic state	Time (min)	*C. reinhardtii*	*E. elegans*
Ultra-oligotrophy	4	67	10
	8	24	9
	16	45	7
	32	65	8
	64	63	5
	128	28	13
Oligotrophy	4	37	6
	8	24	16
	16	19	15
	32	41	9
	64	39	5
	128	28	4
Mesotrophy	4	67	4
	8	60	10
	16	52	3
	32	65	8
	64	83	14
	128	75	21
Eutrophy	4	23	12
	8	29	7
	16	27	10
	32	29	8
	64	24	3
	128	34	19
Hyper-eutrophy	4	18	3
	8	59	13
	16	46	26
	32	16	18
	64	94	8
	128	107	26

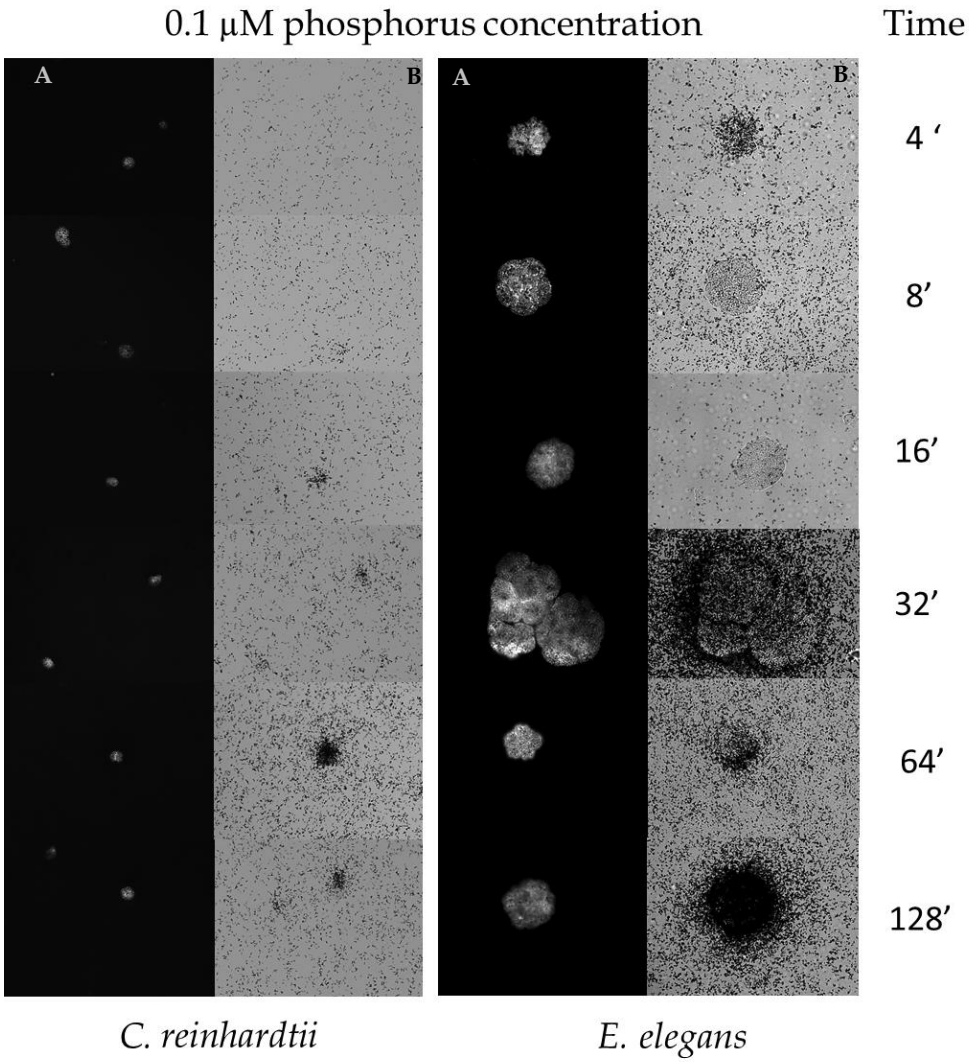


Figure 4.2.3. Sequence of autoradiograms (x63) at different times under 0.1 µM phosphorus concentration. A) epifluorescence microcopy photographs of cell chlorophyll, and B) their corresponding autoradiogram.

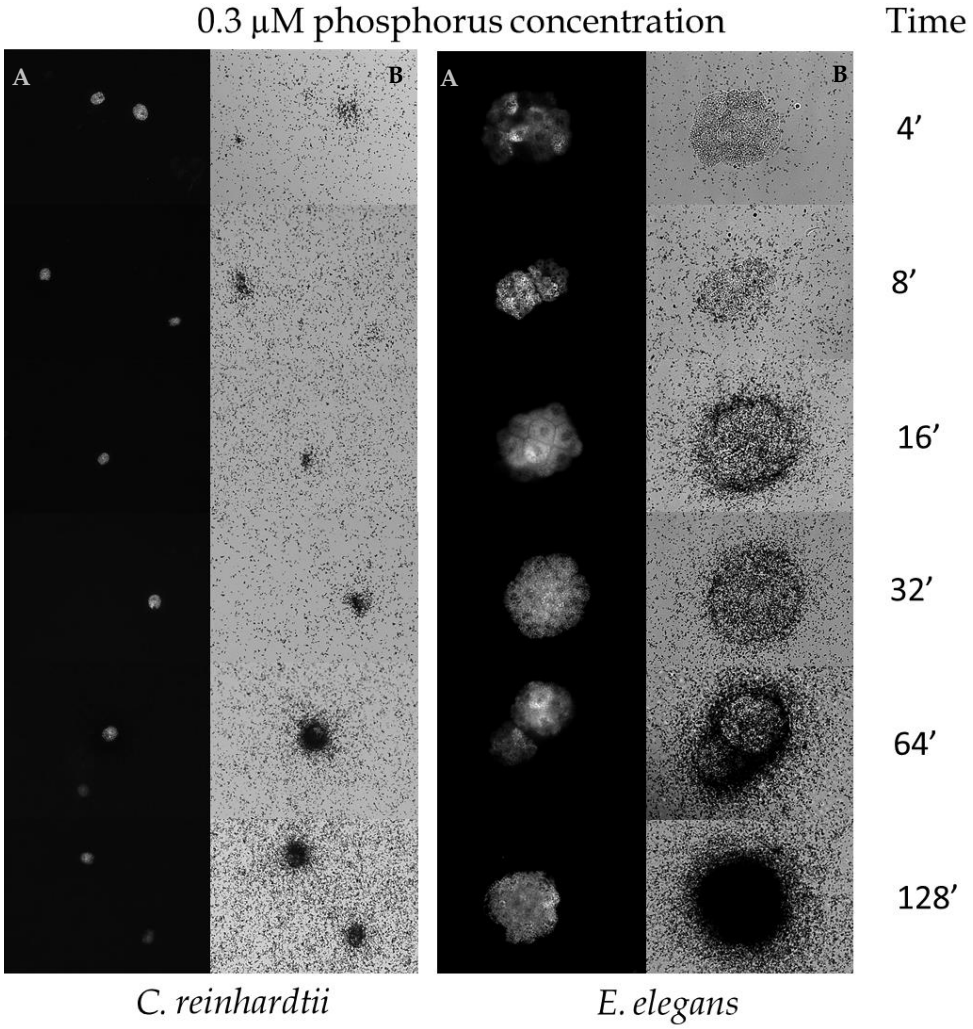


Figure 4.2.4. Sequence of autoradiograms (x63) at different times under 0.3 µM phosphorus concentration. A) epifluorescence microcopy photographs of cell chlorophyll, and B) their corresponding autoradiogram.

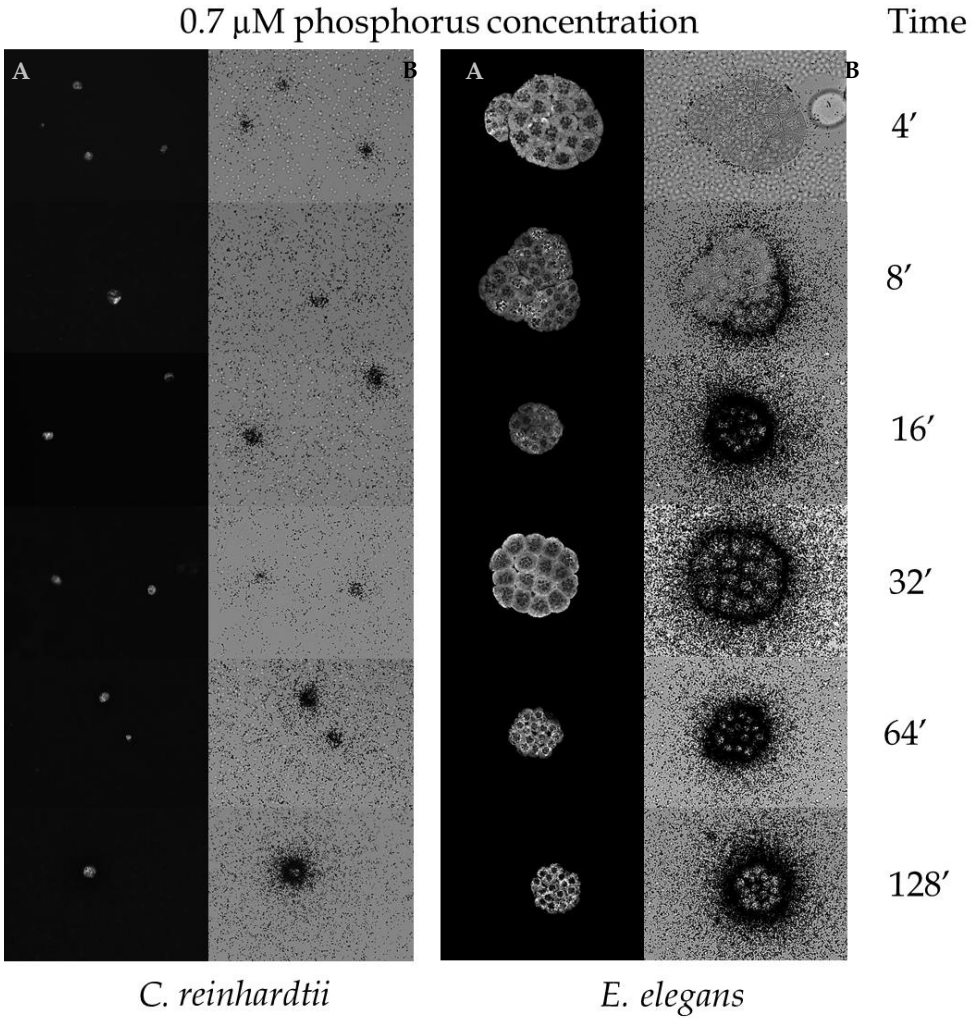


Figure 4.2.5. Sequence of autoradiograms (x63) at different times under 0.7 µM phosphorus concentration. A) epifluorescence microcopy photographs of cell chlorophyll, and B) their corresponding autoradiogram.

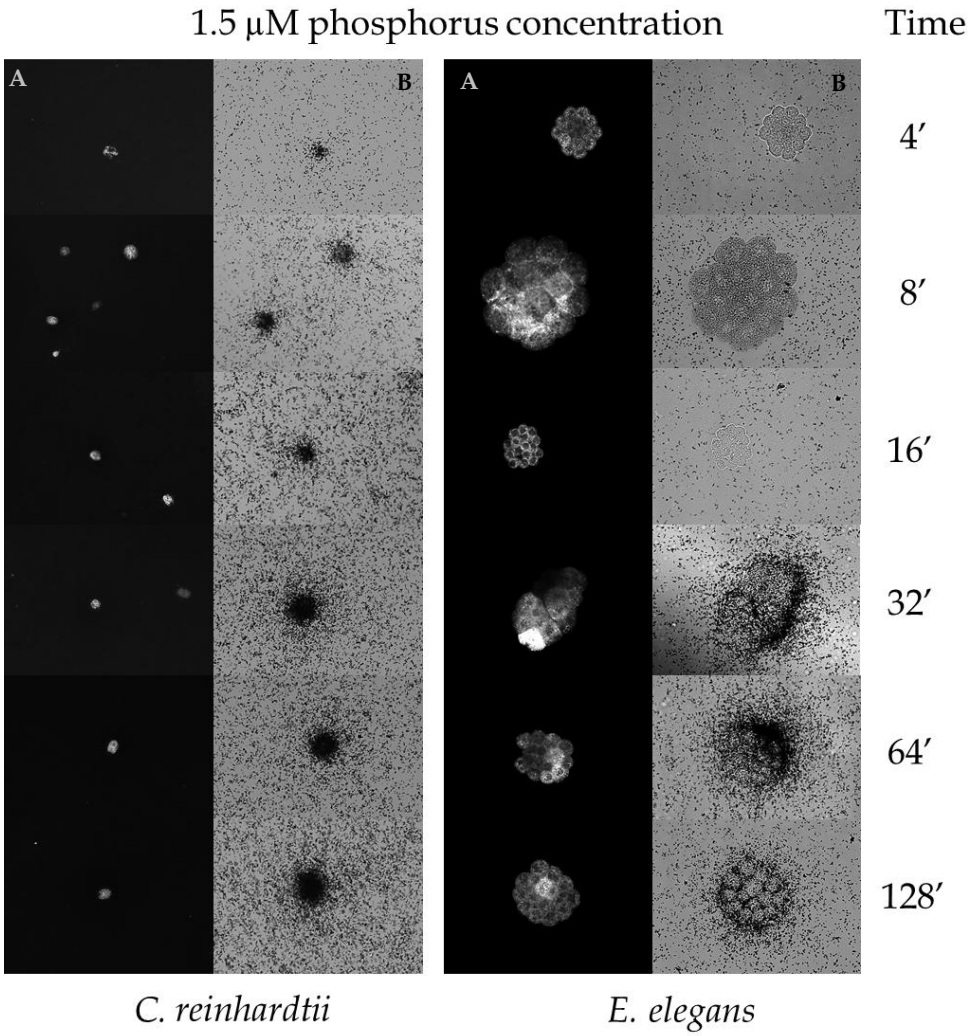


Figure 4.2.6. Sequence of autoradiograms (x63) at different times under 1.5 μM phosphorus concentration. A) Epifluorescence microcopy photographs of cell chlorophyll, and B) their corresponding autoradiogram.

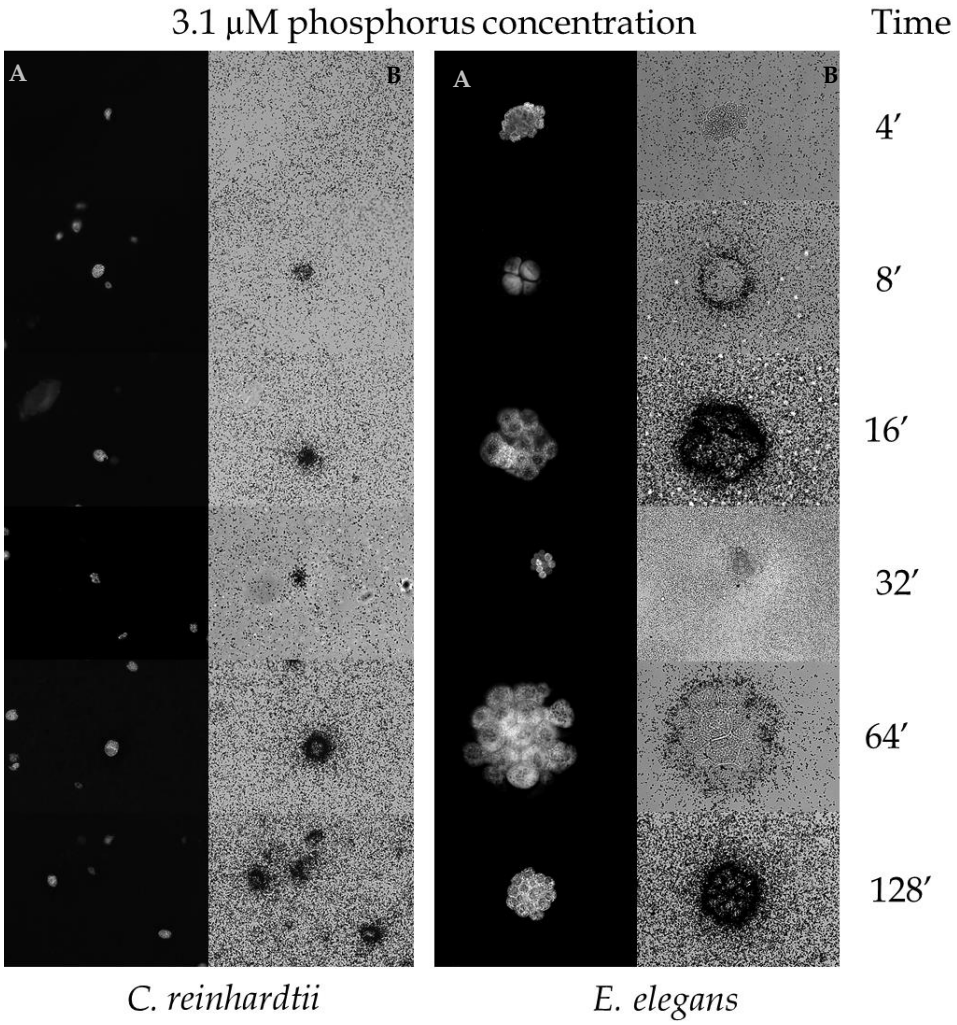


Figure 4.2.7. Sequence of autoradiograms (x63) at different times under 3.1 μM phosphorus concentration. A) Epifluorescence microcopy photographs of cell chlorophyll, and B) their corresponding autoradiogram.

Images from each series time point were analyzed by 'thresholding' (binary contrast enhancement) for the two species. This procedure converts grey-scale images to binary files by defining a grey-scale cutoff point. Grey-scale values below the cutoff become black, and those above become white. This technique favors the detection of the black-stained area edges that surrounds cells and colonies, as a result of radiolabeled phosphate uptake.

Although there is a general increase through time and across concentrations (Fig. 4.2.3-4.2.7), the variability was high from radiogram to radiogram, probably due to small changes in the thickness of the emulsion layer. A radiometric halo was defined around each item for which P uptake was meant to be evaluated. The mean grey were estimated within the halo and in the background, then the values were subtracted, and the remaining value was multiplied by the halo area to obtain and estimation of the P uptaken (in arbitrary units, M ind^{-1}) by cell or colony. The P uptake rate (in arbitrary units, M T^{-1} ind^{-1}) was estimated dividing the latter value by the time at which the labeling was stopped.

4.2.1.4 Size distribution

The area of the organism (cell or colony) was estimated in the epifluorescence image for the same items in which P-uptake was measured. A characteristic length (size) was calculated for each individual using the square root of the measured area. The size range and skewness were larger for *E. elegans* than for *C. reinhardtii* (Fig. 4.2.8), as could be expected. For some purposes, see below, we considered small and large *E. elegans*, that is, above and below 34 µm, respectively; which is the value of the median of its size distribution in the total of samples. There were no significant differences in the size distribution of the species among the experiments performed at the five concentrations (Fig. 4.2.8).

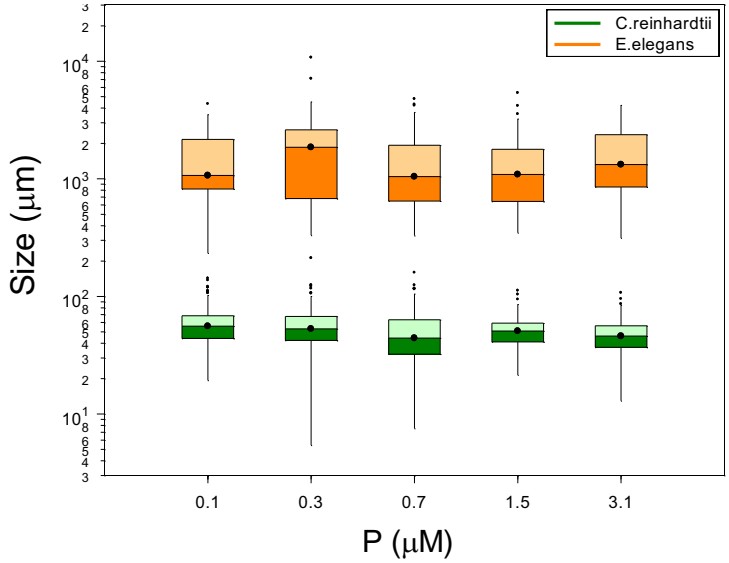


Figure 4.2.8. Size range of *C .reinhardtii* unicells and *E. elegans* colonies across the experimental trophic gradient.

4.2.2 Results

4.2.2.1 Suitable incubations times

Microautoradiography methods tend to be intrinsically noisy, because the procedure is sensitive to small changes in the thickness of the emulsion layer and the variability of the distance of the specimens to the emulsion (Fig. 4.2.9).

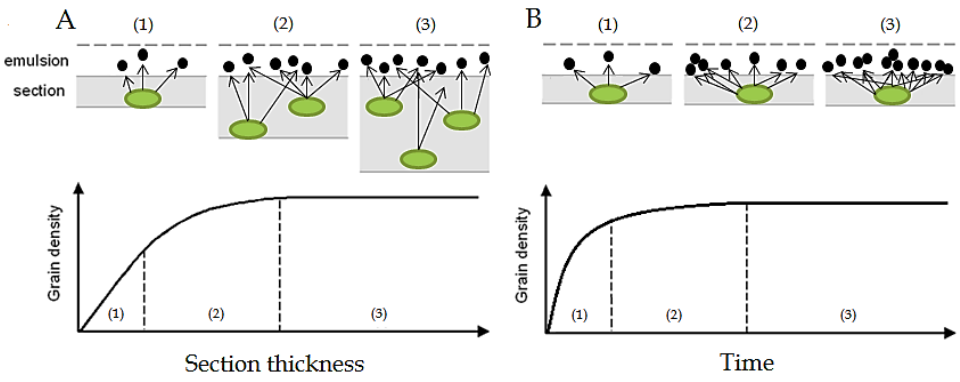


Figure 4.2.9. Vertical sections of a specimen coated with emulsion, showing the effect of increasing section thickness (A), and saturation (B) on grain density.

Beyond minimizing handling variability the influence of these limitations on the results can only be diminish by measuring a large number of specimens. On the other hand, there is an operational limit in the suitable incubation times, because as time increases the likelihood that an emission impinges in the same emulsion place than a previous one increases (Fig.4.2.9 B). Therefore, a first step in the data processing was to determine which time steps where suitable for the calculation of the uptake rates. The apparent total P-uptake increased through time and across concentrations (Figs. 4.2.10, 4.2.11).

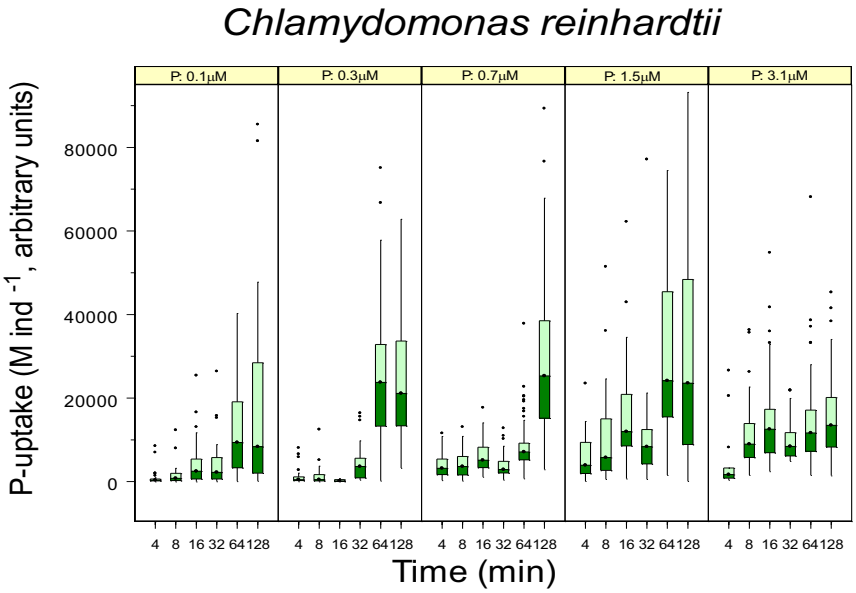


Figure 4.2.10. Distribution of the apparent P-uptake by cell across concentrations for *C. reinhardtii*

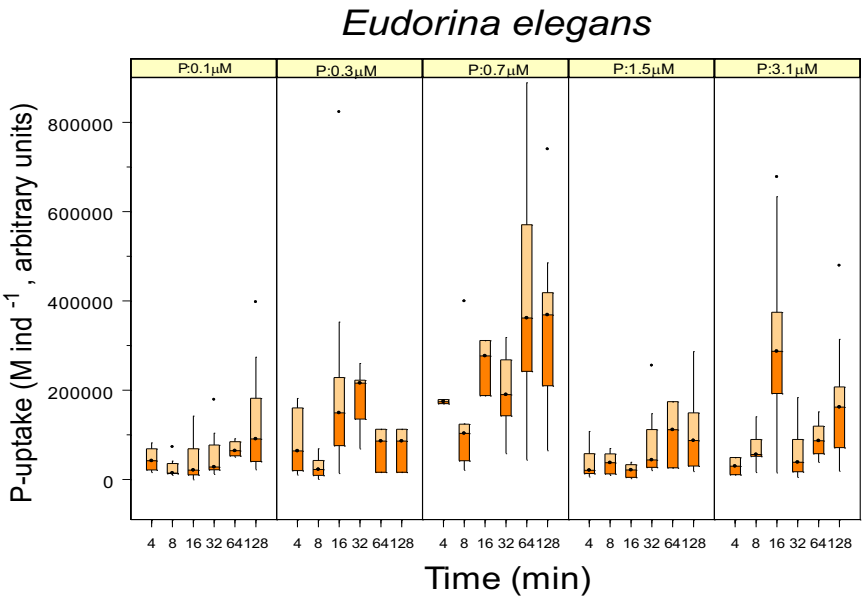


Figure 4.2.11. Distribution of the apparent P-uptake by colony across concentrations for *E. elegans*.

Although scattering was high, there was a plateau in the response beyond 16 min in many cases, which was markedly apparent when calculating the apparent P-uptake rate dividing total P-uptake by the incubation time (Fig. 4.2.12). Beyond 16 min the rates of *C. reinhardtii* and *E. elegans* converged in similar low values indicating that the black yielding effect on the photographic film is no longer proportional to the quantity of radioactive material in the cell. Therefore, that data >16 min was not suitable for the estimation of P-uptake rates and was not considered any more.

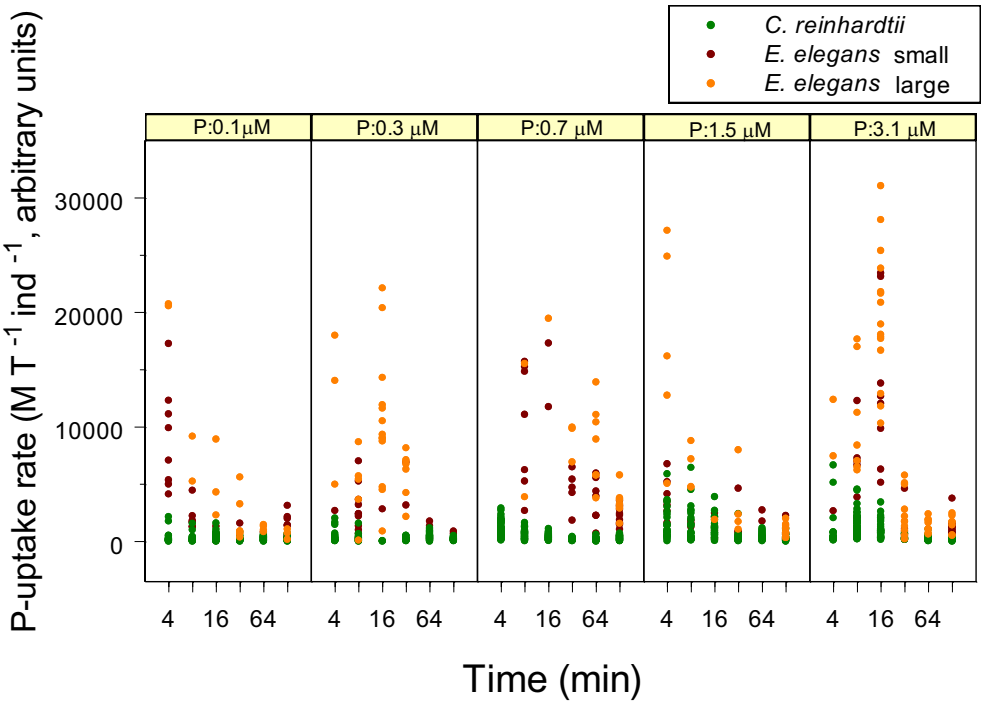


Figure 4.2.12. Effect of time and nutrient concentration on phosphorus uptake rate in a unicellular and colonial phytoplankton population, values declines beyond 16 minutes because of the saturation of the autoradiographic procedure.

4.2.2.2 Phosphorus uptake rates and size

Estimations at 4, 8 and 16 min of the P-uptake rates could be considered population replicates, since the distributions did not statistically differ among them (Fig. 4.2.12). The variability observed was related to the size of the organisms (Fig. 4.2.13). Linear relationships between log-transformed size and P-uptake rates were significant at each of the experimental concentrations (Table 4.2.4). However, the slopes, as well as the dispersion of data, tended to decline as the phosphorus availability in the media increased (Table 4.2.5). Additionally, the intercepts tended to be significantly different from 0, as the availability of phosphorus in the media increased, which indicates a certain amount of P-uptake rate per individual independent of its size. As a consequence, uptake rates for the small *C. reinhardtii*, and *E. elegans* colonies become similar as phosphorus was more abundant in the media. In summary, the size effect on P-uptake rates declines with increasing P concentration in the media and also the variability among individuals of similar size.

Table 4.2.4. Linear regression coefficients for log-transformed P-uptake rates and phytoplankton size across a gradient of P concentrations in the media.

P concentration (μM)	R^2	Intercept	p-value	Slope	p-value	Res. Standard Error	Df
0.1	0.4169	0.1579	0.2173	0.9950	0.000*	0.6029	342
0.3	0.5738	0.1421	0.2616	1.0895	0.000*	0.5887	248
0.7	0.3598	1.2090	0.000*	0.7166	0.000*	0.4983	458
1.5	0.2198	1.80030	0.000*	0.4659	0.000*	0.5453	220
3.1	0.4257	1.2159	0.000*	0.7264	0.000*	0.5326	429

Table4.2.5. Comparison of the linear regression coefficients from table 4.2.4 according to the Potthoff method (see Annex III). There are significant changes in the intercepts and the slopes of the regressions.

F-Test	SS_{Reg}Full	SS_{Reg}Red	(f-r)	MSE Full	F-value	p-value
Coincidence	426.0119	352.2127	8	0.3012	45.567	<<0.01
Intercepts	462.0119	375.0149	4	0.3012	72.2089	<<0.01
Parallelism	462.0119	439.2097	4	0.3012	18.926	<<0.01

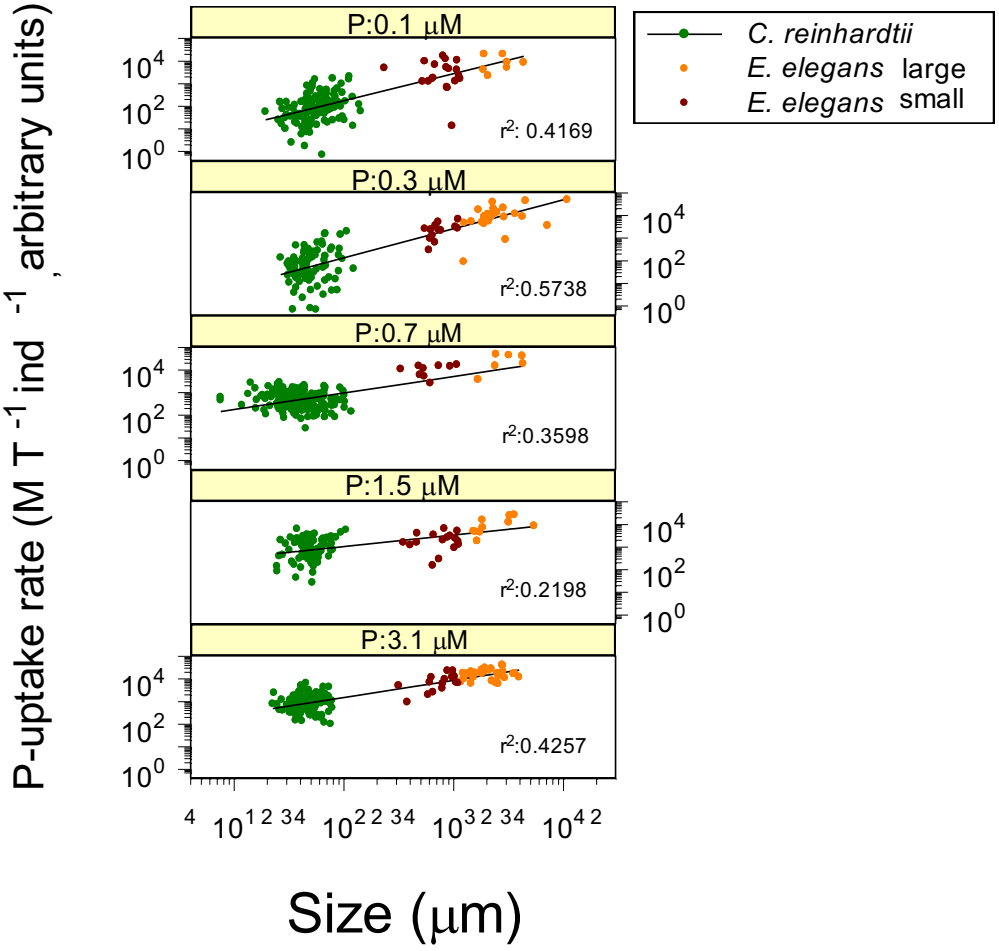


Figure 4.2.13. Relationship between size and P-uptake rate across phosphorus concentration in the media.

4.2.2.3 *P-uptake rates per unit of volume*

P-uptake rates per unit of volume showed a decline with increasing size (Fig.4.2.14). The increase of R^2 compared to Fig. 4.2.13 is certainly spurious because size is also part of the calculations of the P-uptake per unit of volume. However,

the plots are useful to visualize the higher efficiency (supply vs demand) of the small organisms under any P concentration, which are difficult to appreciate without normalizing by size as in Fig. 4.2.13.

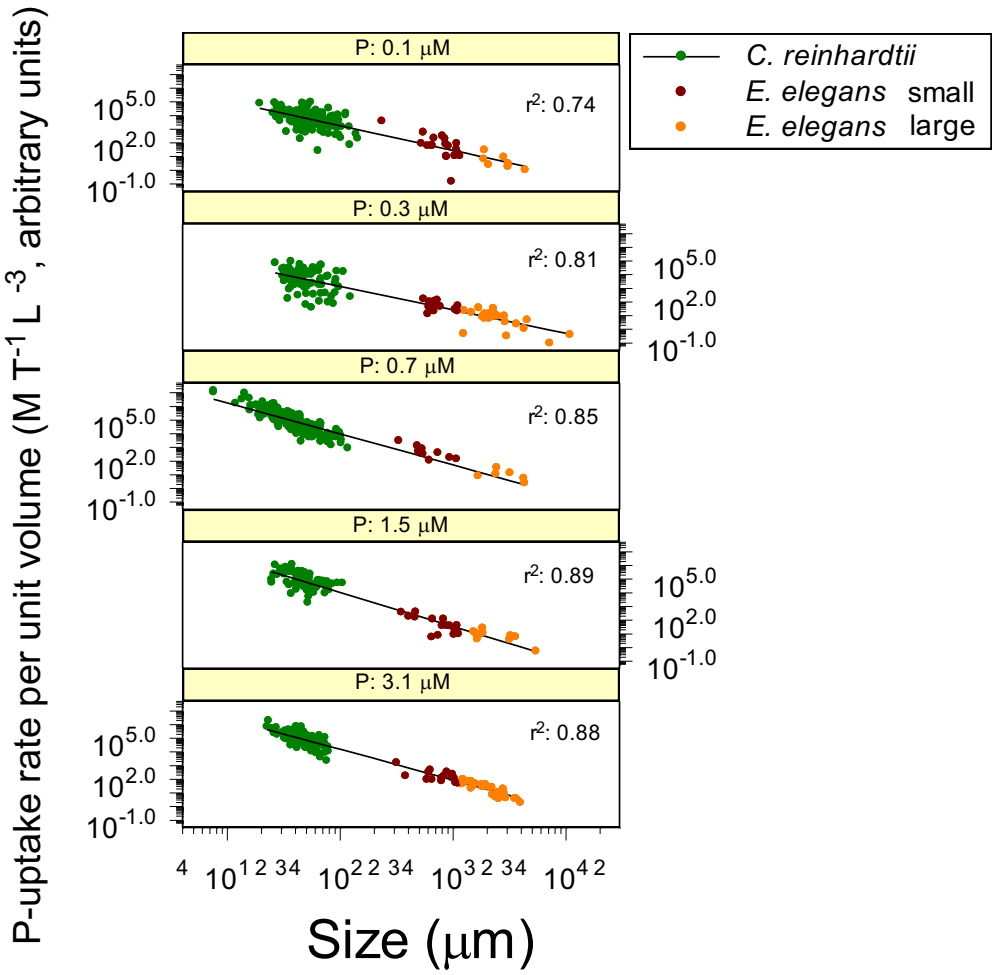


Figure 4.2.14. Body size and P-uptake rate per unit volume relationships across phosphorus concentration in the media.

The dependency of P-uptake rates per unit of volume on size indicates that comparisons of bulk averaging P-uptake rates could be conditioned by differences in the size distribution in each sample. Fig. 4.2.15 compares bulk P-uptake rates per unit of volume with the average values considering the sum of uptake by each single organism. The bulk (traditional method) tends to overestimate the values.

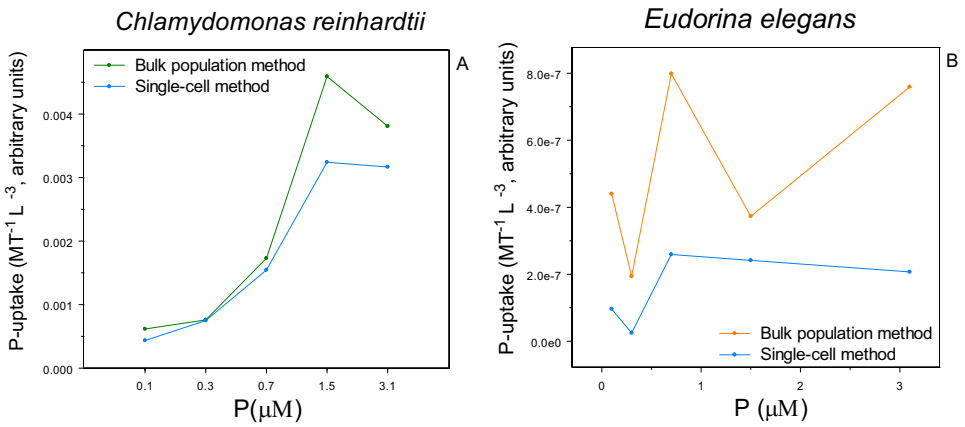


Figure 4.2.15. Phosphorus uptake rate per unit volume comparison for *C. reinhardtii* and *E. elegans*

4.2.3 Discussion

Phytoplankton cells need to reach a minimum nutrient quota to divide, and this specific need is a function of their size. Colonial organisms require that each cell achieve the respective quota if they have to divide synchronously. Our results indicate that the P-uptake size scaling between *C. reinhardtii* unicellulars and *E. elegans* colonies are quite similar (Fig. 4.2.13). As consequence, the P-uptake rate per unit of volume is larger in the smaller organisms. That yields *C. reinhardtii* some advantage over *E. elegans*, and small cells or small colonies, in the respective species, have some intra-specific advantage over large ones. This is in agreement with current knowledge (Falkowski & Oliver 2007). Interestingly, the P-uptake dependence on size declines as its concentration increases. On the one hand, intercepts of the P-uptake *vs* size relationships depart significantly from zero (Table 4.2.4) for P concentrations >1 μmol L^{-1} in the media, indicating that part of the uptake is independent from the cell size. This may indicate that a large

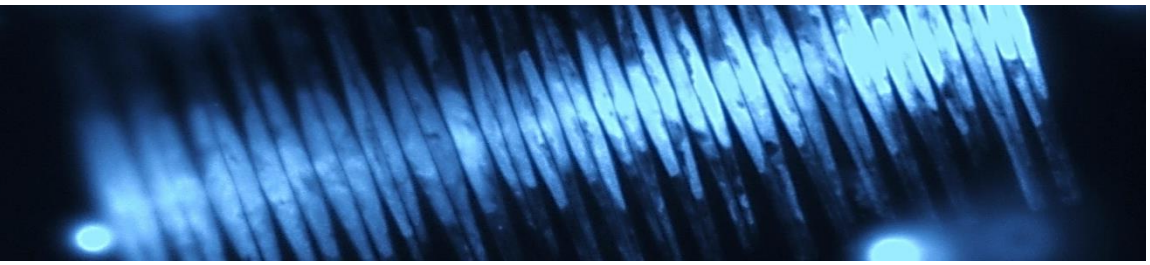
proportion of transport sites at the cell membrane are continuously saturated by P arrival. On the other hand, the slope of the relationship declines as P in the media increases (Table 4.2.4). The change in slope is due to an increase of P-uptake in the small cells rather than in the large cells (Fig. 4.1.13). This may respond to constraints in internal transport in large cells, which do not allow an increase in P-uptake despite higher fluxes towards the membrane. This fact will indicate that there is no real advantage in being large in eutrophic and hypereutrophic environments as differences in P-uptake per unit of volume between small and large individuals are more marked in these environments in favor of small cells. The proliferation of chlorophyte colonies (such as *Eudorina*) in nutrient- rich freshwater systems is, therefore, not a matter related to nutrient efficiency.

Finally, our results show a decrease in intra-population variability as nutrients increase. At the oligotrophic extreme, the variability among individuals of the same species and the same size is larger than at the eutrophic extreme. This may result from a higher stochasticity in the processes involved as a result of less P molecules in the media. The implication of less intercellular P-uptake variability at higher nutrient concentrations is that there is more potential for synchronized cell divisions and, eventually, bloom formation because of the exponential growth. Arguably this could be translated to the synchronization of several species as suggested in Doyle and Poore (1974), who pointed to changes in nutrient levels as a paramount factor in synchronizing the development of several species. And oppositely, oligotrophic systems will be prompt to oscillations in populations in a more random way between species (Massie *et al.* 2010).

For the specific case of colonies, particularly for those that maintain certain morphology, as in many chlorophytes, synchronized cell division is an advantage (if not a strict requirement). According to our results, this synchronization is better achieved in nutrient rich environments, at least for what concerns P-uptake. Therefore, in this aspect, eutrophic systems will be more suitable for colonial phytoplankton.

4.3

Chapter 3: Contrasting phosphatase activity between colonial and unicellular phytoplankton



"We do not place especial value on the possession of a virtue until we notice its total absence in our opponent"

(Friederich Nietzsche)

4.3.1 Introduction

Production of extracellular phosphatases by phytoplankton has been suggested as a response to phosphorus deficiency in both algal cultures (Healey & Hendzel 1979) and natural populations (Healey & Hendzel 1980). However, release of enzymes to cell-surrounding water is largely inefficient as any other nearby organism may take advantage of their activity. It can be hypothesize that this shortcoming may be less drastic for colonial forms. There are at least two reasons for that. First, the colony structure guarantees that there are several cells from the same species very close to the release point; second, the presence of the mucilaginous sheets surrounding many colonies may retain the enzymes preventing diffusion far away from the cell source.

This study aimed to compare extracellular phosphatase activity among freshwater phytoplankton species communities using the fluorogenic substrate ELF. The technique allows the observation of the expression of extracellular phosphatases at the level of single organisms. The study was based on three surveys carried out throughout the growing season (April, August and September) in reservoirs covering a range of different trophic and environmental conditions.

4.3.1.1 Study sites

Samples of the photic zone from seven reservoirs built along the course of Duero River, in the provinces of Zamora and Salamanca, Western Spain (Table 4.3.1), were collected in April, August and September of 2011. Phytoplankton and main nutrients and physical characteristics have been monitored for the last decades by the Hydrogeographic Confederation of Duero (www.chduero.es) in these sites.

Table 4.3.1. Location and some characteristics of the reservoirs studied, (photographs and data from www.chduero. es)

Águeda Reservoir	
	Coord.:40°31'48''N, 6°28'54''W
	Year of construction: 1931
	Total capacity (hm^3): 24
	Area (ha): 177
	Province: Salamanca

Saucelle Reservoir	
	Coord.:41°02'50''N, 6°48'15''W
	Year of construction: 1956
	Total capacity (hm^3): 12.404
	Area (ha): 589
	Province: Salamanca

Aldeadávila Reservoir	
	Coord.:41°12'43''N, 6°41'09''W
	Year of construction: 1963
	Total capacity (hm^3): 714,3
	Area (ha): 364
	Province: Salamanca

Table 4.3.1. (Continuation).

Villalcampo Reservoir	
	Coord.:41°29'28''N, 6°05'07''W Year of construction: 1949 Total capacity (hm^3): 66 Area (ha): 445 Province: Zamora

River Burguillos Reservoir	
	Coord.: 40°23'56.5''N,6°26'55.4''W Year of construction: 1956 Total capacity (m3): 100.000 Area (ha): 3.62 Province: Salamanca

Ricobayo Reservoir	
	Coord.:41°31'39''N, 5°59'06''W Year of construction: 1936 Total capacity (hm^3): 1.200 Area (ha): 5855 Province: Zamora

Table 4.3.1. (Continuation).

Castro Reservoir	
	Coord.:41°34'32''N, 6°11'014''W Year of construction: 1952 Total capacity (hm^3): 26 Area (ha): 180 Province: Zamora

4.3.1.2 Physicochemical measurements and analyses

In the field, some variables were measured with profilers (pH, conductivity (Cond), temperature (Temp), turbidity (Turb), and oxygen (O_2) concentration) and water transparency using Secchi disk depth (Secchi). Two-liter water samples were collected for chemical analyses in each reservoir: one liter was directly stored and the other filtered (0.2 µm GF/F Whatman filters). The filtration system and the bottles were stored in a 5% HCl bath overnight and the filter discs were previously rinsed with MiliQ water before being used. The samples were kept at 3-5ºC until they arrived to the laboratory, where they were stored at -20ºC until analysis.

All samples were analyzed for nitrite (NO_2^-), nitrate (NO_3^-), silicon (Si), chloride (Cl^-), sulfate (SO_4^{2-}), and alkalinity (Alk) by ion chromatography or capillary electrophoresis. Ammonium (NH_4^+, was determined by molecular spectrophotometry by the indophenol method (Fresenius *et al.* 1988). Soluble reactive phosphorus, total dissolved phosphorus and total phosphorus (SRP, TDP and TP, by green malachite spectrophotometry with previous perchloric acid digestion for TDP and TP (Camarero 1994).

4.3.1.3 Phytoplankton samples

Integrated plankton samples were taken using a 4-m tube sampler at four sampling points near the dam. Samples were fixed with Lugol's solution in 100 mL bottles for their subsequent taxonomic identification by laboratories of the Duero

Hydrographic Confederation. Phytoplankton community composition, abundance, biomass and biomass percentage were also calculated.

4.3.1.4 ELF-assay

A volume of 1 L of water from each reservoir was separated for enzyme analyses. We selected 7mL as a suitable volume for incubation according to the trophic state of the systems due to the trophic characteristics of our systems (Gonzalez Gil *et al.* 1998; Dyhrman & Palenik 1999; Nedoma *et al.* 2003; Rengefors *et al.* 2003; Lomas *et al.* 2004). As phosphatases hydrolyzing capacity depends on pH (Jansson *et al.* 1988b), we buffered the samples with 10% Tris-buffer solution (pH = 7.5), in order to perform the assay closer to the historical pH range of the reservoirs (7-8). We used the fluorogenic substrate ELF®97 phosphate (ELFP, 1mL 5mM, contains 2mMAzide, Invitrogen). This soluble substrate is cleaved in the presence of phosphatases into ELF®97 alcohol (ELFA) and forms a fluorescent precipitate (Huang *et al.* 1992). We diluted the stock ELFP solution to 1mM with MiliQ water, and used a final volume of ELF®97 of 0.07 mL to achieve 20µM concentrations in the experiment. Before starting the incubation, we filtered ELF®97 phosphate substrate by 0.2 µm polycarbonate syringe filters (Isopore™ membrane filters, Millipore) to avoid ELFA precipitates in the solution. Then, we firstly added the buffer solution and the natural water aliquots to darkened 8 ml glass tubes, and then 0.07 ml of ELF were added. The cell suspension was incubated for 1.5 hours. The incubations were stopped by gently (<20 kPa of pressure) filtering them by 0.2 µm polycarbonate filters (Isopore™, Millipore). The filters were placed on a bed of absorbent paper to dry the remaining liquid for at least 2 hours. Then, they were mounted the filters on microscope slides adding a drop of Citifluor Ltd mounting media for fluorescence to avoid bleaching. Once labeled, they were stored in a chamber at -20ºC until the microscope processing.

4.3.2.5 Alkaline phosphatase activity evaluation

ELFA precipitates were assessed with an eplifluorescence microscope Zeiss Axio Imager, with a 120 X-cite lamp, using a set of appropriate filters for ELF®97 (EX325-275, BS400, and EM515-565), and a coupled camera AxioCamMrm with an acquisition software AxioVision. Both green ELFA fluorescence and red chlorophyll-a auto-fluorescence were evaluated and the characteristic labeling pattern of each species was reported as digital images.

We evaluated the alkaline phosphatase activity (APA) for each species as the percentage of the total population that was labeled with ELF. We considered the estimation for a species as 'reliable' if, at least, 30 organisms were counted (positively-labeled or not), or when less than 30 organisms were counted but there were more than 3 positively-labeled organisms.

4.3.2 Results

4.3.2.1 Seasonal physicochemical changes

The range of physicochemical and environmental characteristics was relatively broad and the seasonal changes conspicuous (Table 4.3.1, 4.3.2). Water warming was the main feature distinguishing spring form summer conditions. The water temperature in the photic zone changed from 10.3-12.15 ºC in April, to 18.6-24.3 ºC in August, and 18.9-22.1 ºC in September.

To summarize the major patterns of variation, we used a principal component analysis. PCA axis 1 accounted for 45% of the total variance in the data set. This axis summarized the main salinity and nutrient gradient across the reservoirs, only Si concentration varied oppositely to all other compounds (Fig.4.3.1A). The second axis accounted for an 18.5% of the total variance, and reflects the seasonal changes in temperature, water transparency and oxygen, particularly between April and the summer months (Fig. 4.3.1B). Most of the reservoirs followed a similar trajectory in the physicochemical space, the main change was between April and August, and during September the situation was similar to August but reverting slightly towards April conditions (Fig. 4.3.1B).

Table 4.3.1. Physicochemical characteristics of the reservoirs sampled and the percentage of phytoplankton ELF-labeled from the total of individuals observed by reservoir and month.

Reservoir	Month	SRP µmol L^{-1}	TDP µmol L^{-1}	TP µmol L^{-1}	NH_4^+ µmol L^{-1}	NO_2^- µmol L^{-1}	NO_3^{2-} µmol L^{-1}	Si mg Si L^{-1}	Cl^- µeq L^{-1}	SO_4^{2-} µeq L^{-1}
Almendra	Apr.	0.1	0.88	2.2	61	17	2161	50	484	733
Águeda	Apr.	0.08	0.26	0.7	61	4	161	254	64	48
	Aug.	0.08	0.23	0.7	220	1	16	214	79	48
	Sept.	0.15	0.23	0.7	34	3	32	132	96	34
Aldeadávila	Apr.	0.14	0.85	1.9	67	33	2226	164	536	902
	Aug.	0.12	0.32	1.6	405	79	887	71	550	916
	Sept.	0.31	0.45	1.7	11	7	661	71	538	806
Castro	Apr.	0.12	0.3	1.9	133	30	1742	157	448	708
	Aug.	0.67	0.82	1.9	358	112	1048	121	581	1048
	Sept.	0.29	0.41	1.2	353	52	871	89	550	881
Burguillos	Apr.	0.03	0.17	1.8	228	2	48	393	89	19
	Aug.	0.15	0.27	0.9	71	2	32	318	106	8
Ricobayo	Apr.	0.01	0.29	1.8	283	24	903	93	311	300
	Aug.	0.01	0.13	0.7	49	15	323	50	323	304
	Sept	0.06	0.19	0.7	232	7	806	39	353	327
Villalcampo	Apr.	0.07	0.4	1.7	172	28	1645	150	427	620
	Aug.	0.74	1.15	3.6	1119	95	887	46	1099	2579
	Sept.	0.26	0.37	1.3	327	3	871	96	519	822
Saucelle	Apr.	0.75	1.30	1.8	61	39	2000 1081	186	493	808
	Aug.	0.08	0.22	1.4	224	48	677	68	549	945
	Sept.	0.32	0.92	1.7	244	17		86	549	820

Table 4.3.1. (Continuation).

Reservoir	Month	COND µS cm^{-1}	pH	ALK meq L^{-1}	Secchi m	T^a ºC	O_2 mg L^{-1}	TURB NTU	% ELF
Almendra	Apr.	182	7.5	1.08	1.4	11.7	9.9	1.5	0
Águeda	Apr.	32	6.8	0.16	2.0	11.1	8.9	1.4	0
	Aug.	35	7.7	0.20	3.0	24.3	6.2	4.4	0
	Sept.	34	6.7	0.20	1.5	20.3	8.6	0.5	0.5
Aldeadávila	Apr.	303	7.4	1.76	1.0	10.3	11.7	6.6	0
	Aug.	321	8.3	1.70	2.0	22.9	11.4	6.1	0.50
	Sept.	322	7.6	1.78	2.5	20.5	6.9	0.6	0.67
Castro	Apr.	264	7.7	1.82	1.0	10.5	9.3	11.9	0
	Aug.	329	7.3	1.76	3.0	18.6	3.9	6.3	0
	Sept.	330	7.6	1.82	2.0	20.8	6.0	4.1	0.07
Burguillos	Apr.	35	7.5	0.24	2.5	20.4	2.9	15.6	0.33
	Aug.	38	6.5	0.24	1.0	18.9	4.2	7.2	0
Ricobayo	Apr.	186	7.5	1.48	0.6	12.2	11.1	3.5	0
	Aug.	211	7.9	1.42	3.6	22.9	9.1	1.5	0.66
	Sept	223	8.3	1.54	2.4	222	8.9	0.8	0.47
Villalcampo	Apr.	258	7.5	1.80	0.8	10.4	9.5	9.4	0
	Aug.	564	7.6	2.26	2.3	23.9	6.4	2.7	0
	Sept.	326	7.7	1.88	2.0	21.3	5.7	2.1	0
Saucelle	Apr.	289	7.4	1.92	1.4	10.6	11.6	4.4	0
	Aug.	345	8.3	2.00	1.5	21.5	9.5	6.5	0.31
	Sept.	314	8.4	1.78	1.2	21	7.5	1.2	0.09

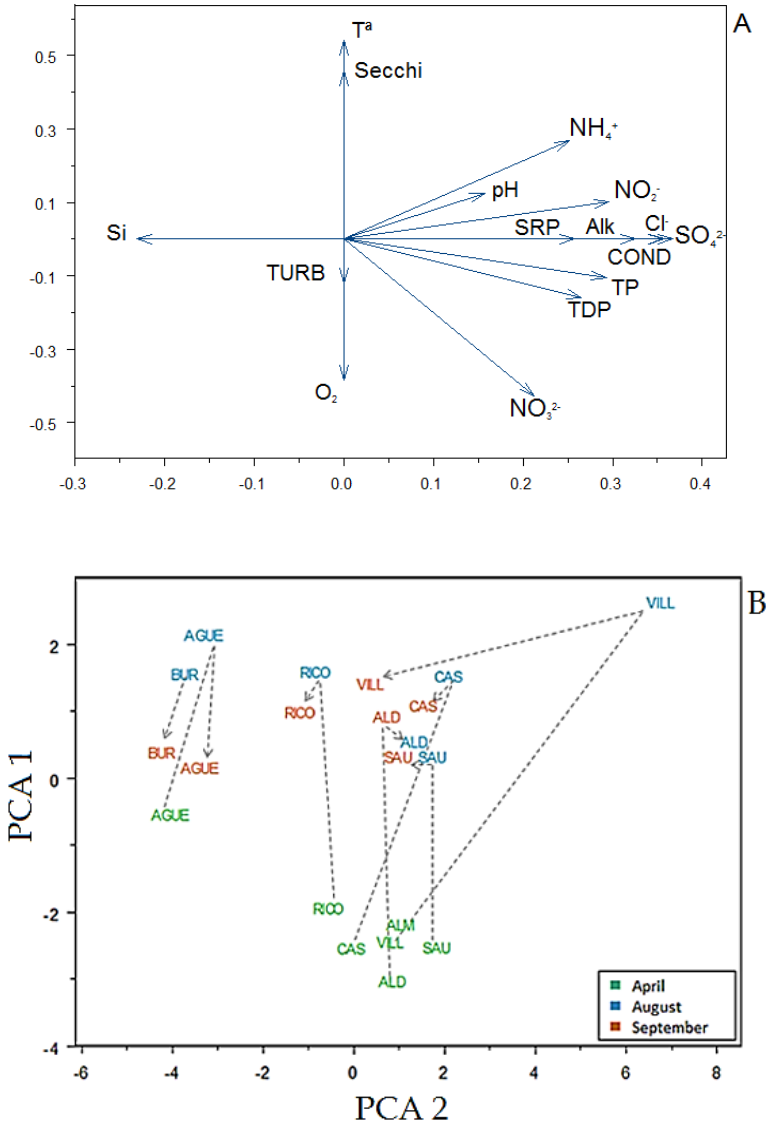


Figure 4.3.1. Principal component analysis of the physicochemical changes. A, loadings of the environmental variables. B, sample scores with indication of the seasonal trajectories followed by each reservoir.

4.3.2.2 Seasonality in alkaline phosphatase activity

There were no samples showing AP activity in April; positive ELF labeling was found in August and September, when 4 out of 7 reservoirs showed ELF positive phytoplankton species. From the principal component analysis, we selected TDP and temperature as representative variables of the two main axes, respectively. And, in order to test the capacity of these variable to discriminate conditions adequate to APA, we classified the samples into ELF positive or negative, and performed a discriminant analysis with Temp and TDP as independent variables, and ELF as the grouping variable. Results showed that from the 21 samples, 19 were correctly categorized into the two possible ELF-activity groups. Plotting APA (ELF) in a Temp-TDP space, we found that temperatures above 20ºC and TDP concentrations below 0.6 µmolL^{-1}favored alkaline phosphatase activity (Fig. 4.3.2).

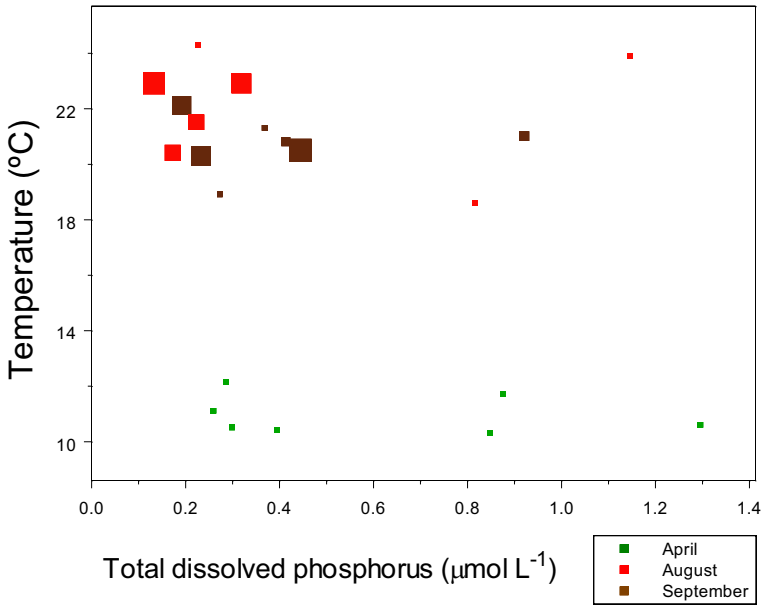


Figure 4.3.2. Alkaline phosphatase activity (ELF labeling) on a total dissolved phosphorus and water temperature framework. Square size is proportional to the ELF response (percentage of ELF-labeled individuals over the total observed in the samples).

4.3.2.3 Species-specific alkaline phosphatase activity

A list of the taxa encountered in the seven reservoirs and their ELF activity are compiled in Table 4.3.3. The observed taxa included representatives of Bacillariophyceae, Primnesophyceae, Dinophyceae, Cryptophyceae, Chrysophyceae, Xanthophyceae, Chlorophyceae, Euglenophyceae and Cyanobacteria.

Table 4.3.3. (Four pages). List of the species observed in eight classes of phytoplankton during the study in the different reservoirs (AGU, Águeda; ALD, Áldeadávila; BUR, Burguillos; CAS, Castro; RIC, Ricobayo; SAU, Saucelle; VILL, Villalcampo).ELF activity is specified by colors (black: unlabeled; yellow: poor-labeled; green: strongly-labeled).

Taxonomic group	**Species**	**Reservoirs**						
		AGU	ALD	BUR	CAS	RIC	SAU	VILL
Bacillariophyceae	*Asterionella* sp.						Apr	
	Aulacoseira granulata	Aug/ Sept			Aug/ Sept			Aug
	Aulacoseira sp.	Aug/ Sept		Aug				
	Cyclotella sp.				Aug/ Sept	Aug/ Sept		Aug
	Discostella pseudoestelligera	Aug						
	Fragillaria sp.						Sept	
	Nitzschia sp.				Aug/ Sept		Aug	
	Stephanodiscus hantzschii		Aug					
	Stephanodiscus sp.					Aug/ Sept		
	Tabellaria sp.	Apr/ Aug/ Sept	Apr/ Aug/ Sept	Aug			Apr	Apr
Cryptophyceae								
	Cryptomonas erosa	Aug/ Sept	Aug/ Sept	Aug/ Sept	Aug		Aug/ Sept	Aug/ Sept
	Cryptomonas marsonii		Aug/ Sept					
	Cryptomonas sp.	Aug/ Sept						
	Katablepharis sp.	Aug						
	Plagioselmis nannoplanctica	Aug	Aug	Aug/ Sept	Aug		Aug	Aug

Cyanobacteria								
	Aphanizomenon sp.					Aug/ Sept	Aug/ Sept	
	Aphanocapsa sp.	Aug	Aug	Aug/ Sept	Aug	Aug/ Sept		Aug
	Crucigeniella apiculata						Aug	
	Chroococcus limneticus					Aug/ Sept		
	Dolichospermum sp.			Aug/ Sept				
	Gomphosphaeria sp.					Sept		
	Merismopedia tenuissima							Aug/ Sept
	Microcystis sp.			Aug				
	Nephrochlamys sp.						Aug/ Sept	
	Oocystisparva						Aug/ Sept	
	Pediastrum simplex						Aug/ Sept	
	Snowella lacustris						Aug	
Chlorophyceae								
	Actinastrum hantzschii				Aug			Aug
	Coelastrum reticularum				Aug	Aug/ Sept		Aug
	Coenococcus planktonicus	Sept	Aug/ Sept			Aug/ Sept	Aug/ Sept	
	Coenochloris fotii	Sept	Aug/ Sept	Aug	Aug	Aug/ Sept	Aug/ Sept	Aug
	Cosmarium sp.						Apr/ Aug	
	Crucigeniella apiculata							Aug
	Chlamydomonas sp.	Apr/ Aug/ Sept		Apr/ Aug/ Sept	Apr/ Aug/ Sept		Apr/ Aug/ Sept	Apr/ Aug/ Sept
	Desmodesmus brasiliensis					Aug		
	Desmodesmus communis		Aug/ Sept					
	Desmodesmus costatogranulatus			Abr/ Aug				
	Desmodesmus sp.				Sept			
	Dictyosphaerium sp.	Aug		Aug		Aug		
	Dictyosphaerium tetrachotomum	Aug						

Didymocystis sp.		Aug					
Elakatothrix genevensis						Aug/ Sept	
Elakatothrix sp.							Aug
Eutetramorus sp.		Aug					
Follicularia sp.						Aug	
Follicularia sp.							Aug
Kirchneriella contortum							Aug/ Sept
Kirchneriella irregularis			Aug/ Sept				
Lagerheimia quadriseta				Aug			
Lagerheimia sp.							Aug
Monoraphidium komarkovae	Apr/ Aug	Apr/ Aug/ Sept	Aug/ Sept	Apr/ Sept	Aug/ Sept	Apr/ Aug/ Sept	Apr/ Sept
Nephroclamys subsolitaria				Aug/ Sept			
Nephrochlamys subsolitaria							Aug/ Sept
Oocystis marsonii		Aug					Aug
Oocystis parva		Apr		Apr			
Pediastrum duplex				Aug/ Sept			
Pediastrum simplex		Aug/ Sept				Aug	
Pediastrum boryanum		Sept					Apr/ Aug/ Sept
Scendesmus communis							Apr/ Sept
Scenedesmus arcuatus				Aug/ Sept			
Scenedesmus communis		Apr/ Aug					
Scenedesmus dimorphus	Apr						
Scenedesmus ellipticus		Aug		Apr/ Sept		Apr/ Aug	Apr/ Sept
Schroederia setigera		Aug		Aug		Aug	
Staurastrum sp.		Apr/ Aug/ Sept				Apr/ Aug/ Sept	
Tetraedron minimum		Aug/ Sept		Aug/ Sept		Aug	
Tetraedron sp.				Aug/			

				Sept			
	Tetrastrum staurogeniaeforme			Aug/ Sept			
	Volvox globator				Sept		
Chrysophyceae							
	Dynobryon sp.	Aug					
	Mallomonas sp.		Aug				
Dinophyceae							
	Ceratium sp.				Aug/ Sept		
	Dolichospermum sp.				Aug		
	Gymnodinium sp.	Aug					Aug
	Peridinium sp.						Aug
Euglenophyceae							
	Trachelomonas sp.			Aug			
	Trachelomonas volvocina		Aug				
Prymnesophyceae							
	Chrysochromulina sp.	Aug				Aug	
Trebouxiophyceae							
	Crucigeniella apiculata	Sept					
Xanthophyceae							
	Goniochloris mutica			Aug			

The species showing APA were a reduced number of those present in the samples (Fig. 4.3.3) and the percentage of individuals ELF-labeled varied from 0.38 to 85.4%. The taxa with observed APA included representative of the Chlorophyceae, Bacillariophyceae, and Cyanobacteria. The extent and frequency of ELF-labeling in the Chlorophyceae was recurrent and important. In contrast, it was limited in Cyanobacteria, for which only some *Dolichospermum* sp., filaments and isolated *Gomphosphaeria* sp., (Fig. 4.3.4.G) colonies were found with ELF-labeling. The only diatom showing APA was *Tabellaria* sp., (Fig. 4.3.4.E). Nevertheless, *Tabellaria* showed a high APA compared with the rest of the phytoplankton community in the reservoir, with almost all colonies showing activity (Fig. 4.3.3).

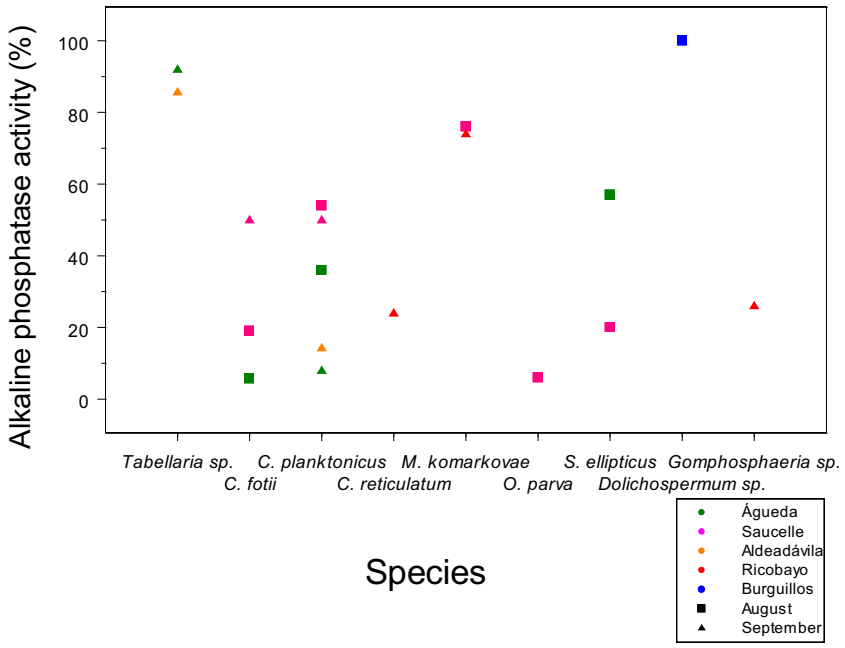


Figure 4.3.3. Species showing alkaline phosphatase activity from August (squares) to September (triangles).

All the phytoplankton species showing APA were colonial, with the exception of *M. komarkovae* (Fig. 4.3.4.F). Interestingly, for the mucilaginous colonies as *Coenococus planktonicus* (Fig. 4.3.4.B), *Coenochloris fotii* (Fig. 4.3.4.C) and *Coelastrum reticulatum* (Fig. 4.3.4.J), not only the cell surface but also the mucilage appeared strongly labeled. Some *Volvox globator* and *Gomphosphaeria* sp., colonies showed APA as well (Table 4.3.3).

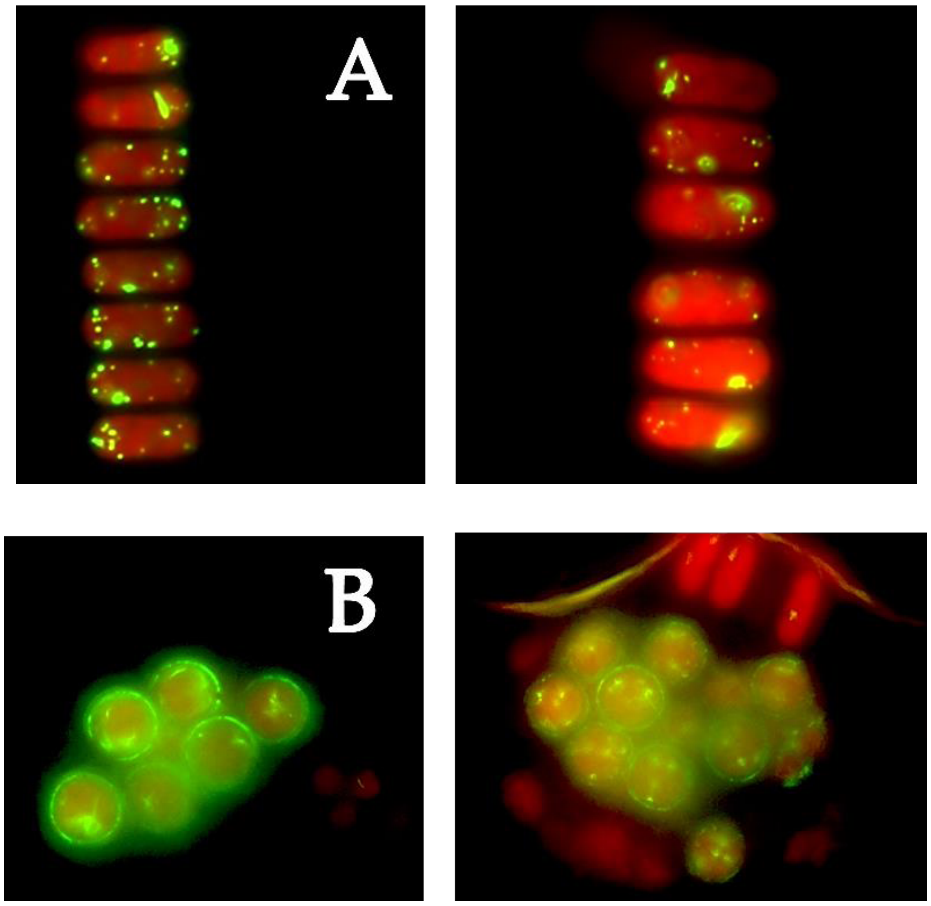


Figure 4.3.4. Images of ELF labeling of alkaline phosphatase activity in natural phytoplankton species/genera using epifluorescence microscopy. A-*Scenedesmus ellipticus*; B- *Coenococcus planktonicus*; C- *Coenochloris fotii*; D- *Oocystis parva*; E- *Tabellaria* sp. ; F- *Monoraphidium komarkovae* ; G- *Gomphosphaeria* sp.; H- *Pediastrum duplex*; I- *Volvox globator*; J- *Coelastrum reticulatum*.

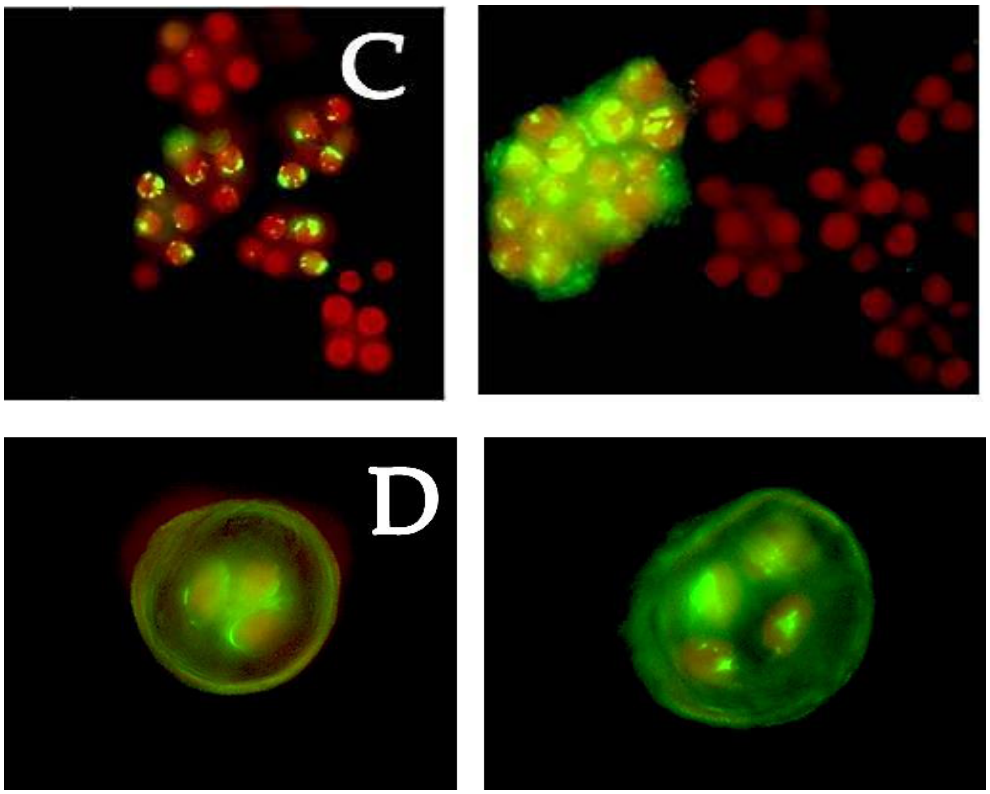


Figure 4.4.3. (Continuation).

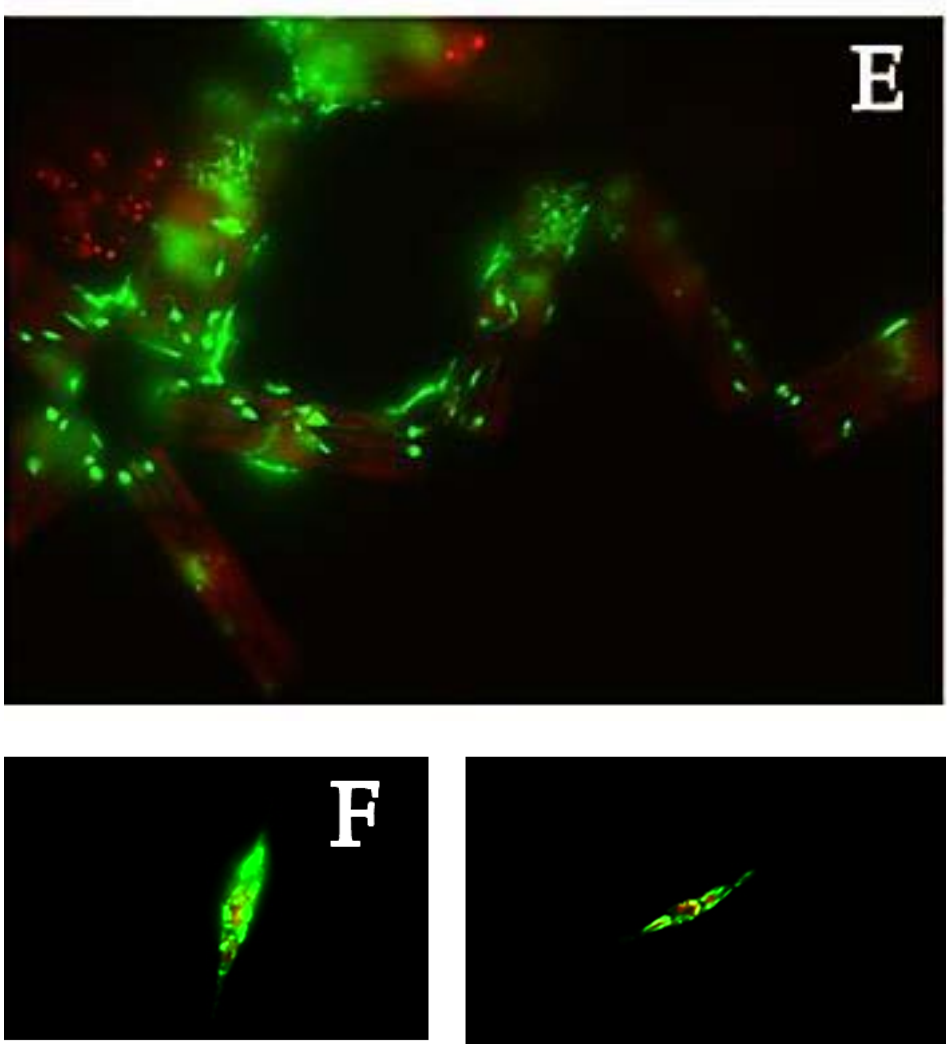


Figure 4.3.4. (Continuation).

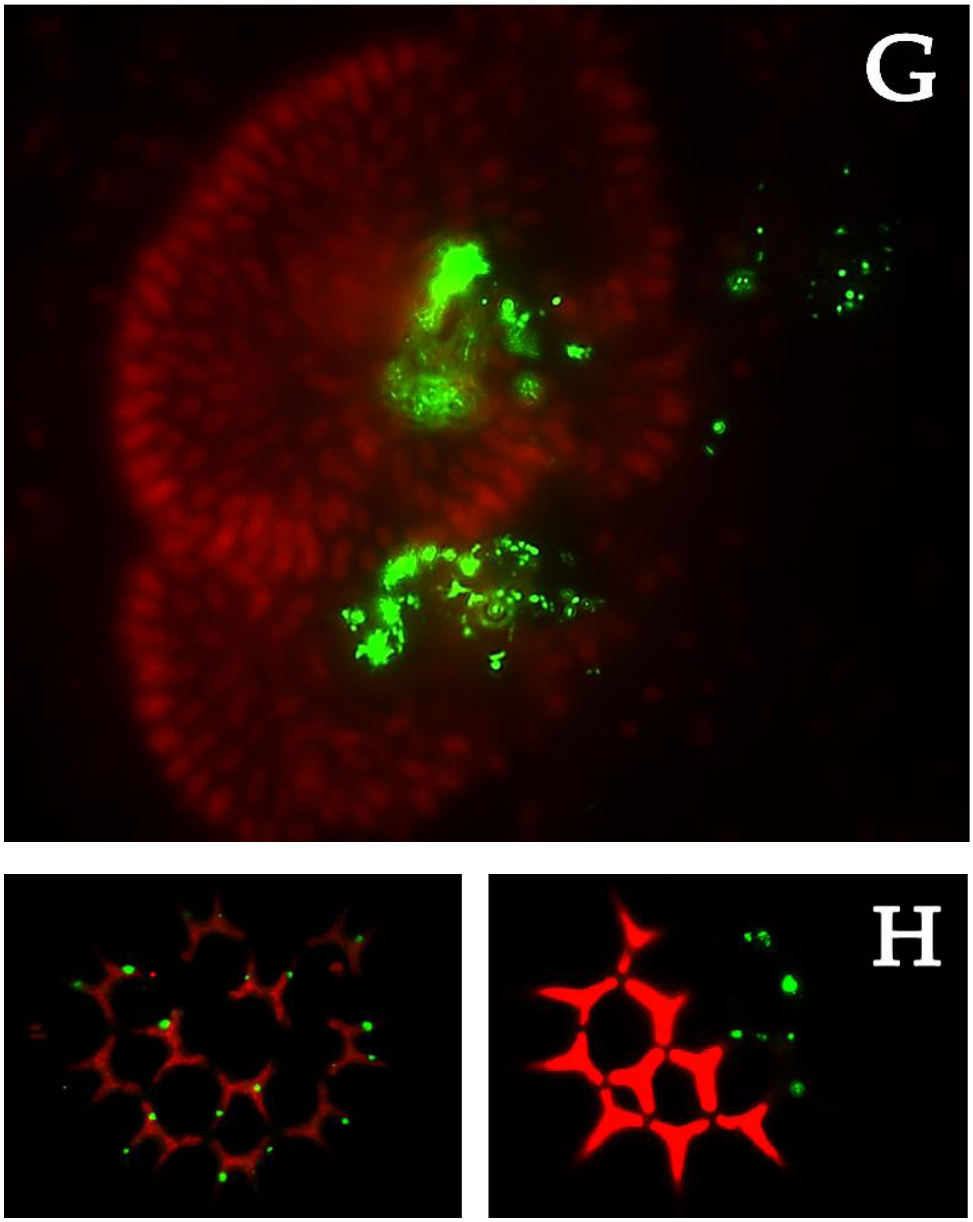
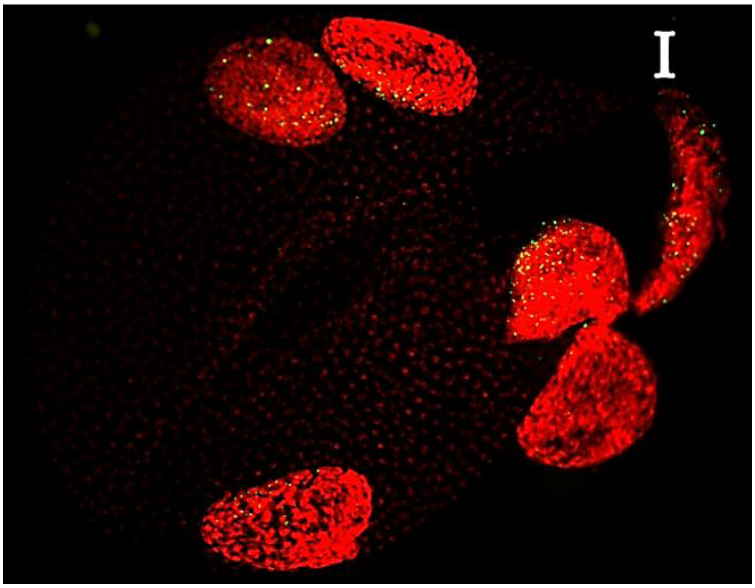


Figure 4.3.4. (Continuation).



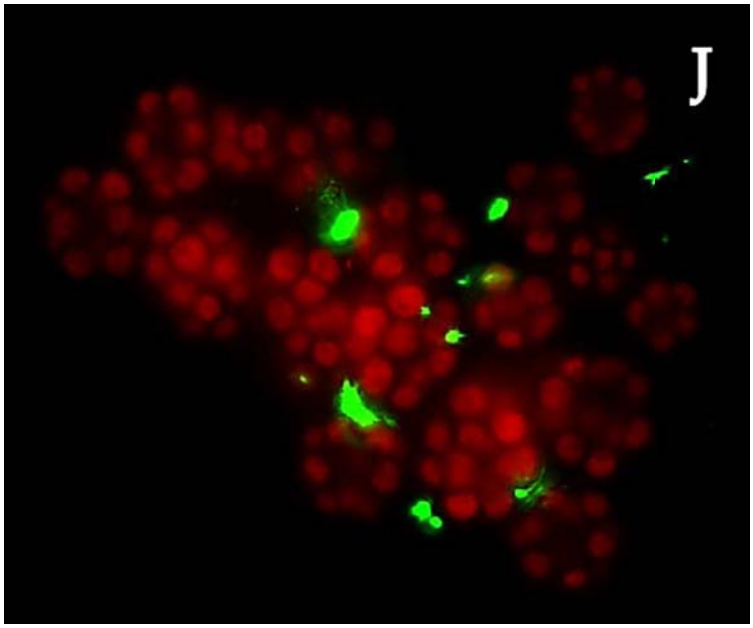


Figure 4.3.4. (Continuation).

4.3.3 Discussion

Among the wide range of inorganic nutrients that phytoplankton organisms require for their living, phosphorous is frequently growth limiting (Karl 2000). The production of extracellular phosphatases can hydrolyze dissolved organic phosphorus compounds in situations where dissolved phosphate is below demand. The use of the ELF assay combined with fluorescence microscopy facilitates studying the extracellular alkaline phosphatase activity (APA) at species level.

The negative APA result for every reservoir in April indicates that populations were not P-limited during this early phase of the growing season. In contrast, during the early summer period, when TDP concentration declined, accompanied by higher water temperatures, specific community phosphatase activity indicated phosphorus limitation: 4 out of 7 reservoirs studied presented a considerable proportion of their phytoplankton ELF-labeled. The late summer period was characterized by a slight increase in TDP concentrations and a marked decline of water temperature. Although APA in September remained high, the number of species producing phosphatases was lower compared to August, and the extent of the ELF-labeling (in all cases but in *Tabellaria* sp.) was less intense.

Most phytoplanktonic species achieve their maximal specific growth rates in the temperature range of 25-35ºC (Reynolds 2006). Therefore, it makes sense to assume that the phytoplankton communities studied were under favorable thermic conditions for growing during August. This would lead to a higher demand of dissolved inorganic phosphorus to meet the algae growth requirements. That would trigger a situation of P limitation, particularly as biomass (and thus higher P demands) accumulated, which eventually would explain both, the decrease of the inorganic phosphorus pool in the reservoirs, and the massive phosphatase production. These findings are in accordance with other studies (Štrojsová *et al.* 2003), which documented extracellular phosphatase activity in a eutrophic reservoir during consecutive seasons. In that study, there was no phytoplankton ELF-labeling in early spring and during the clear-water phases in all the years sampled, and they found species of Cyanobacteria, Chlorophyceae and Conjugatophyceae showing phosphatase activity mainly in summer and at the

beginning of autumn. Therefore, the seasonal patterns were quite similar to our results.

There were marked seasonal differences in phosphatase activity between taxa. For diatoms, we found phosphatase activity only in the genus *Tabellaria* sp. This genus is characterized by forming long and heavy filaments that performs their usual habitat attached to substrata (Flower & Battarbee 1985), and that hardly remain in the pelagic for long time. The use and derivation of water during summer in the reservoirs probably could have provoked turbulent water regimes, which favored the apparition of this species in the upper water layers where, independently of what the inorganic phosphorus limitation was they started to release phosphatases to cover their phosphorus physiological needs. In addition, other studies have suggested a high nutrient affinity relative to large cell sizes in diatoms (Thingstad *et al.* 2005), a fact that could explain organic phosphorous hydrolysis for *Tabellaria* sp.

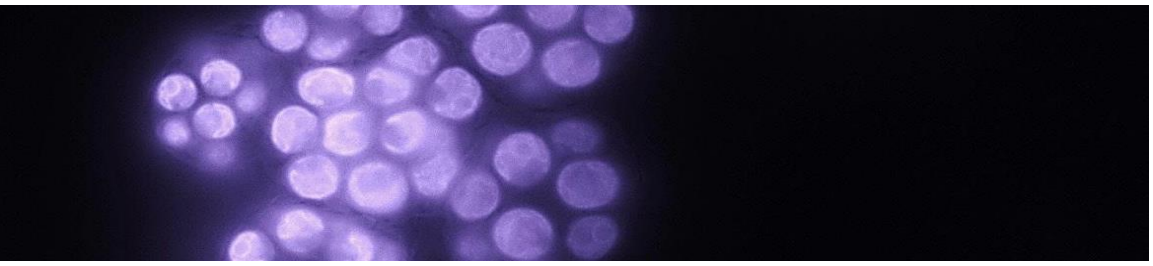
In the case of Cyanobacteria, only two species (*Dolichospermum* sp. and *Gomphosphaeria* sp.) were found ELF-labeled, the first in August, and both in September. *Dolichospermum* sp., ELF staining pattern was characterized by dots in the cell surface, whereas *Gomphosphaeria* sp., presented accumulation of ELF in the colonial central cavity. In our samples, cyanobacteria colonies were poorly labeled. Other studies point to a low phosphatase production in cyanobacteria relative to their luxury consumption of phosphorus, which is in accordance with the hypothesis that, as cyanobacteria usually grow in nutrient-depleted systems, they might require long periods of phosphate stress before inducing phosphatases production (Kulaev *et al.* 1983; Reynolds 1984). Our results support this idea, as the number of species found ELF-active doubled in September, as well as the ELF-signal of labeled organisms. In September, these species had been nearly a month more under phosphorus scarcity conditions.

Chlorophyceae species were an exception to the general lack of phosphatase activity observed in the samples. Most of ELF-labeled species in the summer period were green algae. With the decrease of TDP and the increase of temperatures in August, several species of Chlorococcales that conformed a large percentage of the representatives of this order in the samples, were heavily marked with ELF precipitates. This result points to an enhanced extracellular phosphatase production in periods of phosphorus limitation as a general trait of chlorophytes,

or at least, Chlorococcales. With the exception of *Monoraphidium komarkovae*, the ELF-active species were large mucilaginous colony-forming organisms, which presented strong stain ELF-labeling both located on the cell surface, and in the mucilaginous coverage. The rate of ELF labeling for *Coelastrum reticulatum*, *Coenochloris fotii,* and *Coenococcus planktonicus*, three species with comparable structure and sizes, was similar. It is possible that due to their large sizes, and particular colonial performances (forming distant or branch-like colonial sub-unities), the internal transportation of phosphorus is complicated even in non-scarcity situations, and hence, the release of extracellular phosphatases supposes a strategy to overcome this disadvantage against other competitors.

4.4

Chapter 4: Phytoplankton colonial forms across lake trophic states



"We become what we behold. We shape our tools, and thereafter our tools shape us".

(Marshall McLuhan)

4.4.1 Introduction

On ecological scales, the balance between growth and decaying processes determines the biomass achieved by phytoplankton populations. On evolutionary time scales, phytoplankton life forms may have evolved with higher selective pressure for enhancement of reproduction (growth) or mitigation of mortality (decay). The appearance of any kind of phytoplankton form across the trophic gradient of freshwater ecosystems indicates that there is no a unique optimal evolutionary solution; however, it may happen that certain trophic conditions are more favorable than others to a certain live form.

There is a large variety of shapes and forms among phytoplankters. A very simple classification of this diversity is distinguishing between unicellulars and colonial organisms, and among the latter according their dimensionality (1D to 3D), that is, filaments, plates and globular colonies. Colonies imply an increase in size of the functional unit and the dimensionality of this colony strongly influences the surface to volume ratio of this unit. Surface (S) imposes a constraint on the possibilities of exchange with the surrounding medium, which is vital for osmotroph organisms. Volume (V) determines the demand of resources for growth and eventual duplication. Therefore, S/V ratio have been identified as a main trait in phytoplankton evolution concerning growth (reproduction) selection pressures(Lewis 1976). However, size and shape are also related to decaying processes (e.g., sinking rates, grazing susceptibility) and coloniality may offer new capacities for regulation. As nutrient availability and grazing pressure differ across trophic gradients, we may ask whether any of the four forms described above are particularly successful at some part of the whole trophic range. A large variability can be suspected, because in this simple classification we are not taking into account many other traits that drive phytoplankton life. Therefore, this question can only be investigated using a very large data set including many sites across the whole trophic range.

Here, we explore the biomass statistical distribution of a large number of species of the four phytoplankton forms in a phytoplankton data set compiled for the evaluation of the ecological status of water bodies according to the EU Water Frame Directive. We consider whether these basic types differ in their distribution across the trophic gradient, whether they achieve different standing stock (total

population biovolume), and whether the possible patterns are consistent across the main phylogenetic lineages.

4.4.1.1 WISER Database

In this study we used the phytoplankton database collated in the EU 7th Framework Program project WISER (Moe *et al.* 2013). In particular, we used the species incidence and biomass (biovolume) to characterize the distribution, and total phosphorus (TP) to characterize the trophic state of the lakes. After screening for missing data and unreliable values, a total of 8527 samples from 1734 lakes of 19 European countries were used. Full details of the database can be found in Phillips *et al.* (2010).

We focused on phytoplankton species with a population biovolume > 1µm^3 L^{-1}, and belonging to groups with a substantial number of colonial species, namely: Cyanobacteria, Chrysophyceae, Bacillariophyceae, and Chlorophyceae. Within the latter we only considered two orders, Chlorococcales and Volvocales, which were analyzed separately due to the contrasting living ways (i.e., non-flagellated *vs* flagellated, respectively). The few heterotrophic species from these groups were not considered, neither the benthonic species that occasionally appear in the plankton. Regarding TP, only data spanning from 1to 1000 µg P L^{-1} were included as a safety measure against extreme values that might include mistakes in any step of the compilation and collation process.

4.4.1.2. Statistical analyses

In freshwater ecosystems is a common practice to characterize the trophic state using total phosphorus (TP)(Vollenweider 1968). This is by definition a continuum but it is useful to regard it as a set of discrete states to which we can ascribe certain general characteristics. This discretization in trophic state classes facilitates visualizing the patterns and relationships with other variables, particularly when variability is high and we are dealing with a large number of observations. In our analysis, we have considered four TP classes based on (Wetzel 2001), oligotrophy: (<10 µg P L^{-1}), mesotrophy (≥10 -<35 µg P L^{-1}), eutrophy (≥35 -<100 µg P L^{-1}), and hypereutrophy (≥100 µg P L^{-1}).

The average species biovolume (ASB) was calculated for the populations of each species within each trophic state. The ASB values were used to analyze the distribution of the four morphological types across the trophic state gradient. First, one-way ANOVA analyses were run to test whether the distributions differ among life-forms and across trophic states. If the test was significant, we use generalized linear models (GLM) to relate ASB to TP and life-forms. Log-transformed ASB data to normalize the largely skewed distributions were used. In all the regressions, the χ^2 statistic for each term that entered the selected model to assess whether it provided a significant contribution to the model was calculated. This analytical approach was followed considering the whole phytoplankton data set, and also for each main taxonomic group.

In addition, to explore the distribution of ABS that each life-form achieved under different trophic states, there was also investigated if there were differences in cell size across the trophic gradients and life-forms. We used a cell size classification provided by Giuseppe Morabito (pers. com.), based in classes of log cell volumes. We analyzed the data using box-plots and quantile regressions relating TP and cell size for each life-form class.

4.4.2 Results

4.4.2.1 Life-forms incidence and biomass across trophic states

A total of 2063 phytoplankton species, representing 16 taxonomic classes and 25 orders from WISER database were finally included in our analyses. The data set was numerically dominated by three groups (Chlorophyceae, Bacillariophyceae, and Cyanobacteria), which represented a 68% of the total data. The distinct life-forms had a similar relative species representation across the trophic gradient (Table 4.4.1): unicellulars (ca. 48%), filaments (13%), plates (16%), and globular colonies (23%). The frequency with which a species of certain life form appeared in the samples (community) was always low, particularly at oligotrophy, indicating the high diversity of community assembling that exists (Fig.4.4.1).

Table 4.4.1. Number of species by life-form and trophic state.

Life-form	Oligotrophy	Mesotrophy	Eutrophy	Hypereutrophy
Unicellulars	188	278	280	245
Filaments	47	80	74	64
Plates	68	93	94	81
Globulars	97	131	130	109
Total	400	582	578	499

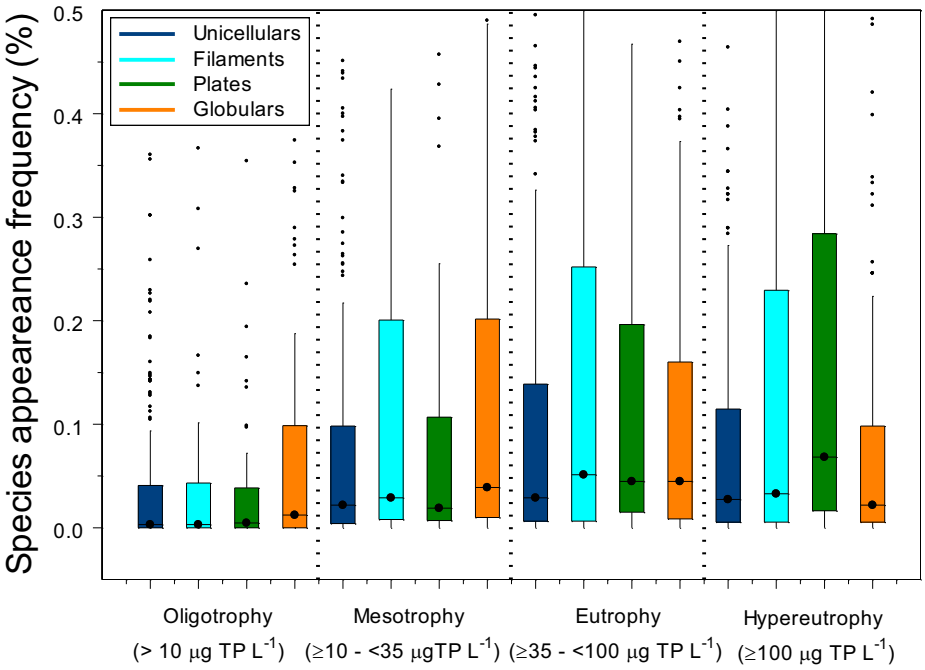


Figure 4.4.1. Frequency of species appearance across trophic gradient by life-forms.

The average species biovolume (ASB) differ across trophic state and life-forms (Fig.4.4.2) (One-Way ANOVA *F*-value 1.646, *p*-value 0.0.55). ABS increases across the trophic gradient, although variability was high within the same life-form. Remarkably, filaments showed outstanding higher ABS than any of the other life-forms at any trophic condition.

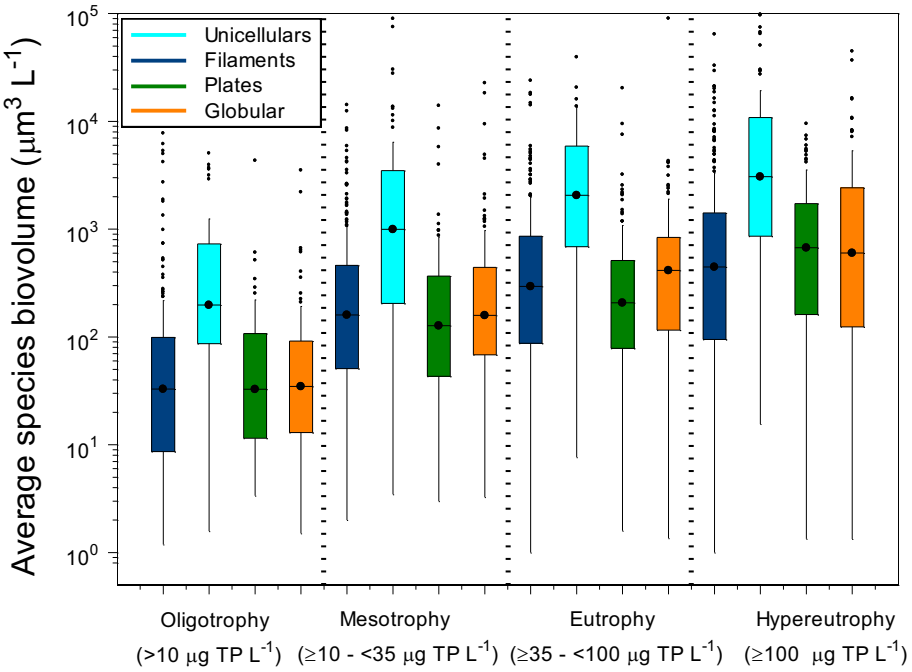


Figure 4.4.2. Life form distribution of the average species biovolume across trophic states.

GLM indicated significant effect on ABS of both, TP and life forms as well as their interaction (Table 4.4.2).

Table 4.4.2. Generalize linear modeling (GLM) relating ASB with TP and life-forms.

Variables	Df	Deviance Res.	Df	Residual dev.	Pr (chi)
TP	1	38.8529	2059	1783.260	4.569743e-010
Life-form	3	147.6718	2056	1635.588	0.000000e+000
TP *Life-form	3	201.5366	2053	1434.052	0.000000e+000

4.4.2.2 Life-forms diversity across trophic states

Filamentous species tend to show larger average population biomass than the rest of the forms (Fig. 4.4.3A), as expected from the results shown in the previous

section. However, when we compare the distribution of species richness across the trophic gradient, filaments follow a similar distribution than unicellulars, whereas plates and globular colonies differ (Fig. 4.4.3B).

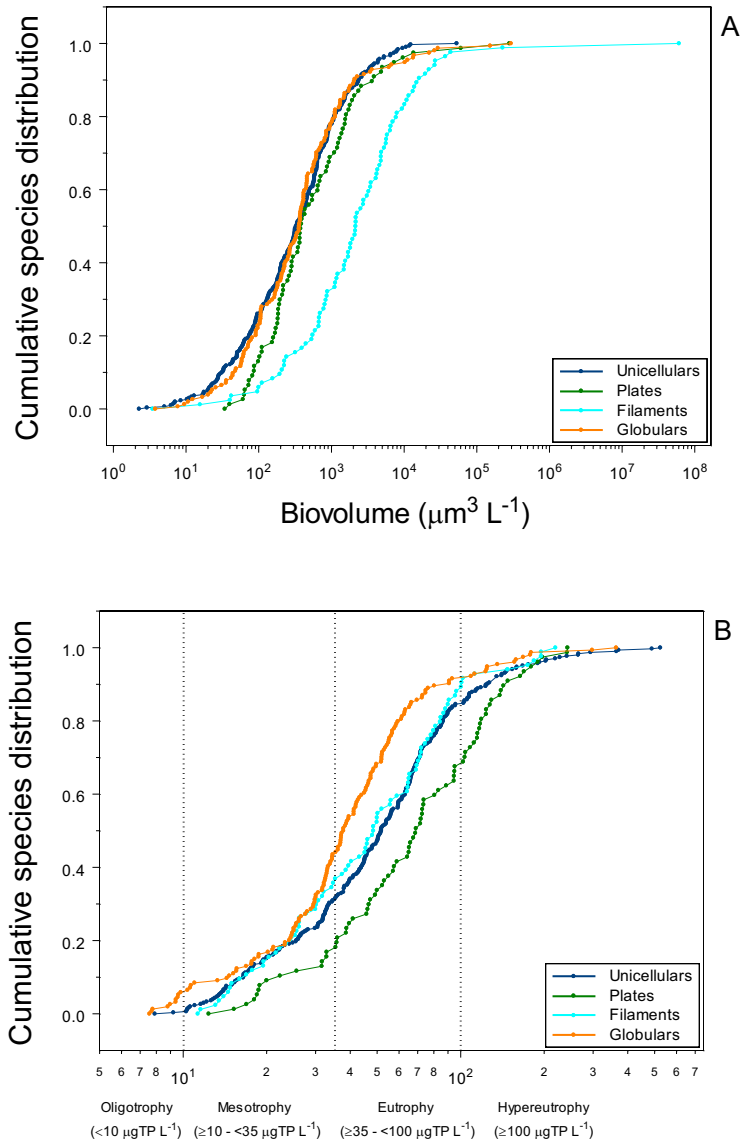


Figure 4.4.3. Cumulative species distribution *vs* average population biovolume (A) and total phosphorus (B).

For plates, no species were found in oligotrophic conditions, the species of this form start appearing at a slower rate than the species of other forms along mesotrophy, and finally they show a steeper increase than the others in hypereutrophy. In the case of globular forms, the performance along the trophic gradients is close to that for unicellulars and filaments, except in eutrophy, in which they increase in number faster than the rest of the life-forms.

4.4.2.3 Life-forms and main taxonomic groups

The general pattern of ASB increase with trophic state was also present and significant in each of the main taxonomic groups (Table 4.4.3).

Table 4.4.3. ANOVAs assessing differences in the distribution of life-forms across trophic states for the main taxonomic groups.

Taxonomic group	Df	Sum of Sq	Mean Sq	F value	Pr (F)
Chrysophyceae	11	33.31751	3.028864	4.440696	0.00001041508
Cyanobacteria	14	122.9629	8.783064	10.69997	0
Bacillariophyceae	11	50.1915	4.562866	6.363063	1.133304e-009
Chlorococcales	11	90.2800	8.207275	15.58842	0
Volvocales	7	10.93842	1.562632	2.758267	0.01951176

According to the GLM analysis both TP and life-forms were significant for determining ASB for most taxonomic groups, with the exception of the Volvocales (Table 4.4.4). In contrast to the general case, the interaction between TP and life-forms was not significant in any of the groups. For Chrysophyceae, Bacillariophyceae and Cyanobacteria the life-forms explained more ASB variability than TP. In the case of Cyanobacteria and Bacillariophyceae, filaments showed outstanding higher biomass that the rest of the forms across all trophic states (Figs. 4.4.4 B, C). Interestingly, the patterns were quite similar in these taxonomic groups, which are phylogenetically extremely distant. In the Chrysophyceae, the diverging life-forms were the globular colonies, particularly in hypereutrophic conditions, which showed always larger biomass than unicellulars and plates (Fig. 4.4.4 A).

Table 4.4.4. GLM results relating ASB with TP and life forms for the main taxonomic groups of freshwater phytoplankton.

Taxon	Variable	Df	Deviance Res.	Df	Res.Deviance	Pr(chi)
	TP	1	11.76445	147	114.9966	0.00
Chrysophyceae	Life-form	2	15.19311	145	99.8035	0.00
	TP*Life-form	2	1.11495	143	98.6886	0.57
	TP	1	39.53053	366	373.1922	0.00
Cyanobacteria	Life-form	3	46.66772	363	326.5245	0.0
	TP*Life-form	3	1.61963	360	324.9049	0.65
	TP	1	7.13653	381	309.0940	0.01
Diatoms	Life-form	2	29.45453	379	279.6395	0.00
	TP*Life-form	2	4.27061	377	275.3689	0.12
	TP	1	58.65995	580	331.7240	0.00
Chlorococcales	Life-form	2	7.29807	578	324.4259	0.03
	TP*Life*form	2	1.86910	576	322.5568	0.39
	TP	1	2.321538	46	31.27795	0.13
Volvocales	Life-form	1	2.097279	45	29.18067	0.15
	TP*Life-form	1	1.852199	44	27.32847	0.17

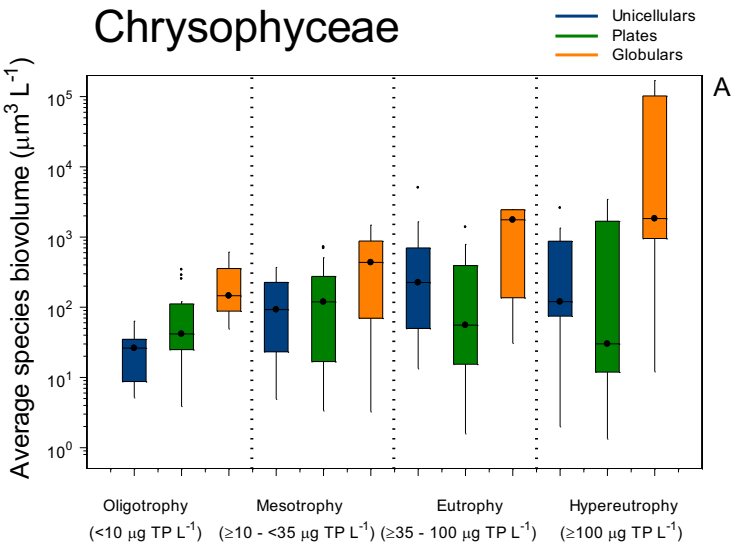


Figure 4.4.4. Average species biovolume across trophic states and among phytoplankton life forms considering the main taxonomic groups individually.

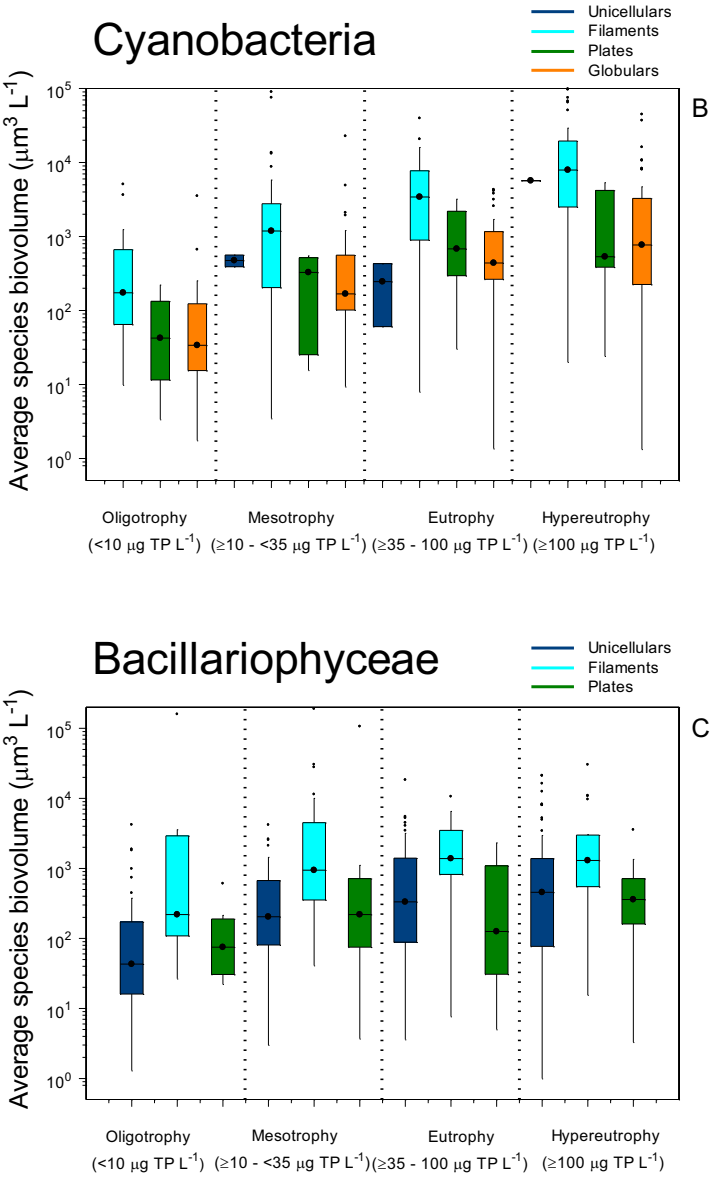


Figure 4.4.4. (Continuation).

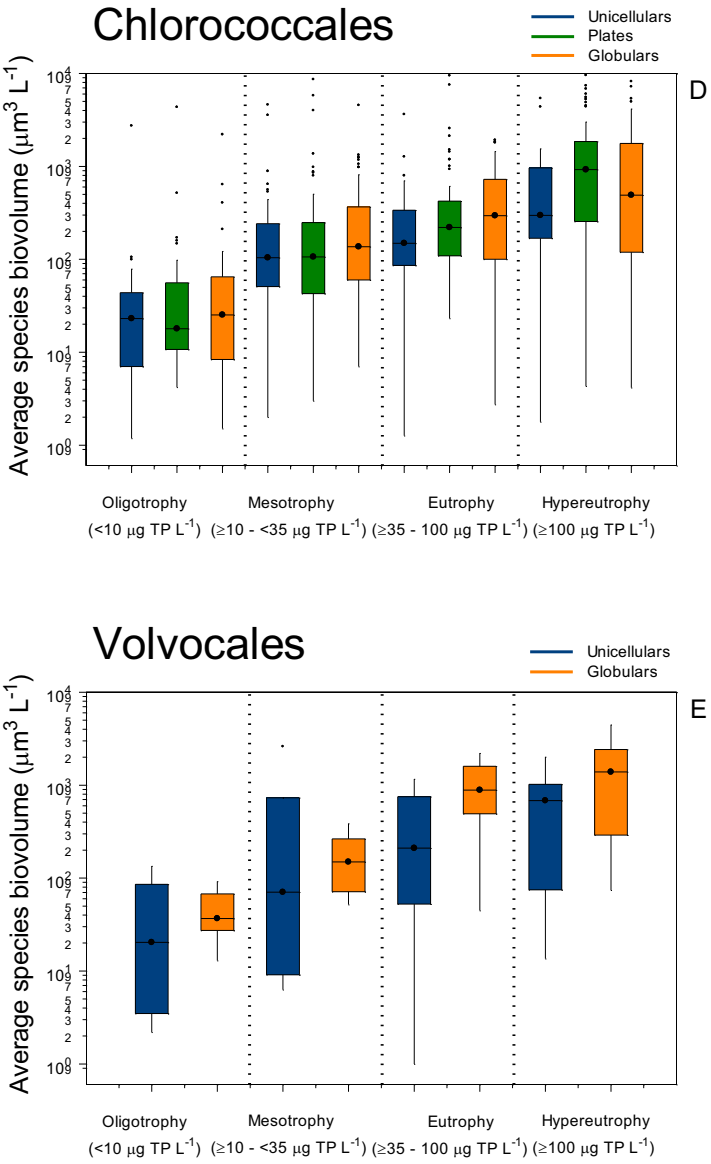


Figure 4.4.4. (Continuation).

4.4.2.4 Life-forms and cell size

Despite a large scattering, cell size was smaller in colonial life-forms than in unicellulars (Fig. 4.4.5) the smallest size was achieved in globular colonies. However, there was not a clear size pattern across trophic states.

Quantile regressions indicated that trophic state influence on cell size was only relevant in unicellular and globular colonies for the species in the low size range (<25 percentile) (Table 4.4.5, Fig.4.4.5). In these latter situations, the higher the trophic state, the smaller the species in the low range of sizes. Thus, to test whether unicellular and globular colonies showed different slopes for the lower limit response (25 percentile), we tested their variances (*F*-value 1.3945, *p*-value << 0.00001), and as no significant difference occurred, we compared their slopes, which resulted different (*F*-value 0.3013; *p*-value 0.74) as visual inspection already suggest (Fig. 4.4.6).

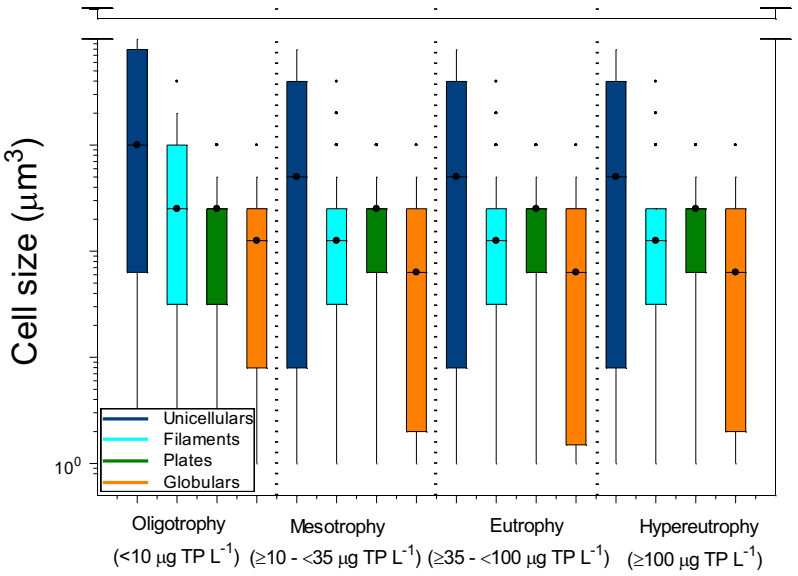


Figure 4.4.5. Phytoplankton mean cell size and life forms across trophic states.

Table 4.4.5. Quantile regressions (25, 50 and 75 percentiles) relating cell size of distinct life-forms to TP.

Quantile	Typology	Coefficient	Value	Std.Error	t-value	Pr (>|t|)
25	Unicellulars	Intercept	1.81	0.38	4.76	0.00
		Slope	-0.003	0.001	-2.21	0.027
	Filaments	Intercept	1.50	0.144	10.38	0.00
		Slope	3.05e-19	0.0013	0.00	1.00
	Plates	Intercept	1.80	0.27	6.68	0.00
		Slope	3.30e-19	0.0011	0.00	1.00
	Globulars	Intercept	0.61	0.28	2.14	0.03
		Slope	-0.00117	0.00082	-1.44	0.15
50	Unicellulars	Intercept	2.70e+00	0.16	17.40	0.00
		Slope	-1.50e-18	0.00093	0.00	1.00
	Filaments	Intercept	2.10e+00	0.15	13.99	0.00
		Slope	1.56e-20	0.00090	0.00	1.00
	Plates	Intercept	2.40e+00	0.00	1.42e+16	0.00e+00
		Slope	4.81e-19	0.00	1.69e-01	8.66e-01
	Globulars	Intercept	2.11	0.13	15.93	0.00
		Slope	-0.0018	0.001	-1.83	0.00
75	Unicellulars	Intercept	3.60	0.073	48.98	0.00
		Slope	-6.29e-18	0.00062	0.00	1.00
	Filaments	Intercept	2.40	0.23	10.51	0.00
		Slope	1.43e-18	0.00087	0.00	1.00
	Plates	Intercept	2.40	0.00	1.18e+16	0.00
		Slope	2.50e-18	0.00	1.04	2.97e-01
	Globulars	Intercept	2.40	0.00404	593.34	0.00
		Slope	-7.97e-19	0.00008	0.00	1.00

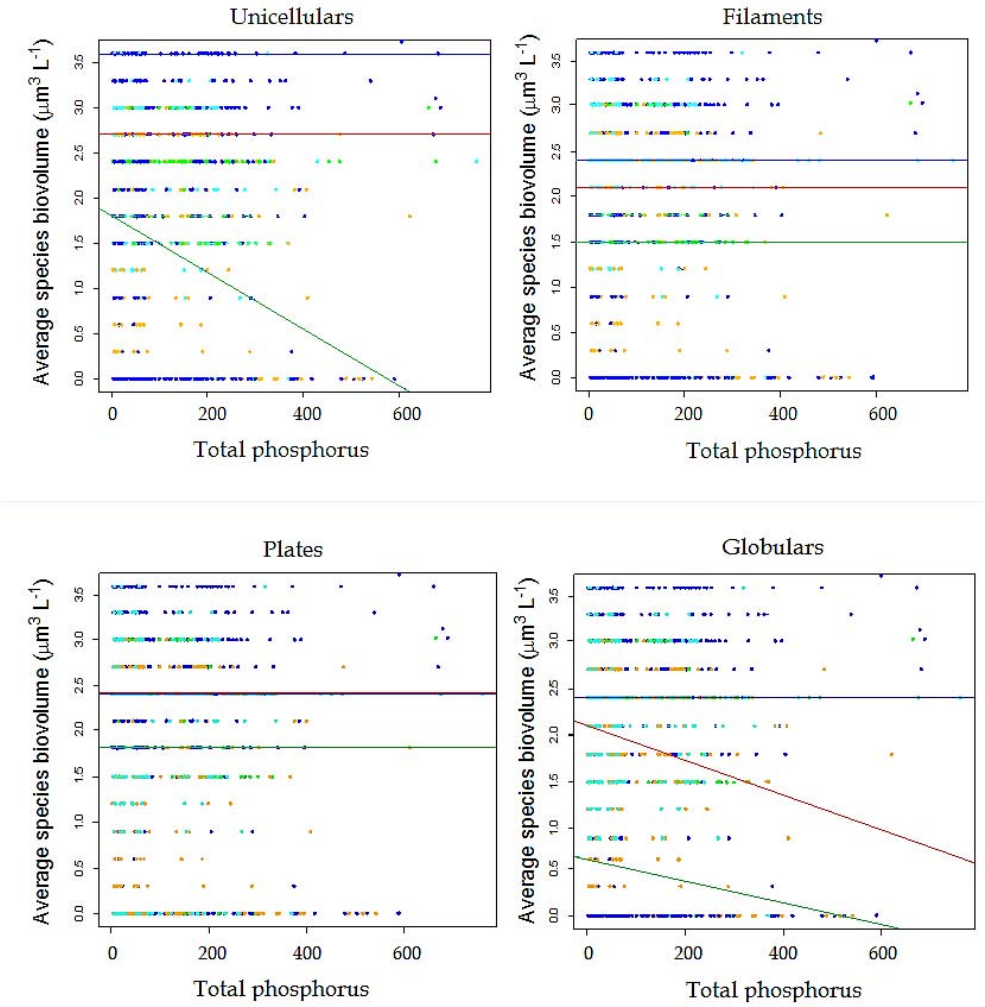


Figure 4.4.6.25, 50 and 75 percentiles for the relationship between TP (µg PL^{-1}) and unicellular, filamentous, plate and globular forms phytoplankton ASB. Color-code: 25 percentile green, 50 percentile red, 75 percentile blue.

4.4.2.5 Mucilage effects

Some colonies are rich in mucilaginous external structures. We investigated whether this is of relevance in the context of biomass accumulation in freshwater ecosystems. ANOVA and GLMs test were performed for each taxonomic group,

but no significant effect was found complementary to the TP or life-form effect. As an example of the lack of relationship, we show the Chrysophyceae case (Fig.4.4.7). There are differences among distributions (ANOVA *F*-value: 18.17037; Pr (F): 0.000036). However, only TP and life-forms are significant for explaining them, and the presence of mucilage, or its interaction with the life-forms, has no influence across the trophic gradient (Table 4.4.6).

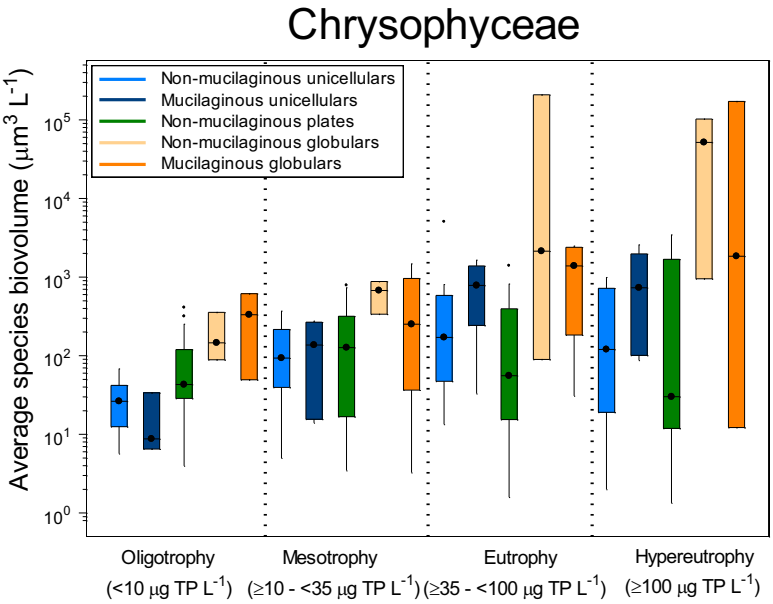


Figure 4.4.7. Mucilage effect on average species biovolume across the trophic gradient and by life-forms.

Table 4.4.6. GLM results for mucilage effects.

Variable	Df	Deviance Res.	Df	Res.Deviance	Pr(chi)
TP	1	11.76445	147	114.9966	0.0006
Mucilage	1	1. 66602	146	113.3306	0.1967
Life-forms	2	13.66165	144	99.6689	0.0011
TP*Mucilage	1	2.03517	143	97.6338	1.5370
TP*Life-forms	2	0.01493	141	97.6188	0.9926
Mucilage*Life-forms	1	3.01952	140	94.5993	0.08227
TP*Mucilage*Life-forms	1	1.79128	139	92.8080	0.18077

4.4.3 Discussion

4.4.3.1 Colonial life-forms and phytoplankton biomass across trophic states

The standing phytoplankton biomass depends on the balance between growth and decay. Phytoplankton growth requires resources (nutrient and light), therefore, generally, the higher the nutrient load to the system, the higher phytoplankton biomass (biovolume) supported. Phosphorus is a key nutrient for phytoplankton primary production and, as a consequence, the trophic state of freshwater ecosystems has been related to total phosphorus concentration (TP). However, the relationship between P and phytoplankton biovolume is not straightforward since there are multiple factors influencing the efficiency of the conversion into algal biomass, including the nutrient stoichiometry and the nature of the species involved. If, instead of the whole phytoplankton community we consider a certain species, then the discrepancy between P in the system and the particular biovolume of the species can be even larger, since the species is interacting with many others. In consequence, we should expect an increase of the average species biovolume (ASB) with increasing TP in the system, but with a large scatter around the main tendency. In fact, this is what our analysis showed (Fig. 4.4.1), for all the life-forms considered, whether colonial or not.

Filaments showed outstanding higher biovolumes than any other life-form at each trophic state (Fig.4.4.1). There is no evidence that cells in filaments can grow at higher maximum rates than unicellulars or other life-forms, therefore, these high biovolumes have to be achieved by either a highly synchronize blooming of the populations, or because decay processes are diminished. There are many filamentous species that, in fact, bloom in the water column under some particular conditions, for instance, *Aulacoseira* species among diatoms and *Planktothrix* among Cyanobacteria (Takamura *et al.* 1992; Steinberg & Trumpp 1993; Rucker *et al.* 1997; Queimalinos *et al.* 1998; Interlandi *et al.* 1999; Salmaso 2002). However, this is not the general case as indicated by Fig. 4.4.1, where filaments are widely present in all the system trophic states. Therefore, lower decay of populations (at least during the growth phase) seems to be the more general explanation. From the decay processes acting during the growth phase of the population, sedimentation and grazing are the two more important ones (Ferguson *et al.* 1982). Filaments show an accelerated sedimentation unless they have mechanisms for enhancing floatability

(such as gas vacuoles in some Cyanobacteria) (Walsby *et al.* 1991). In any case, lower sedimentation is not a common future to all filaments (e.g., high sedimentation rate of *Aulacoseira*). Therefore, reduced sedimentation is not probably the reason for higher biomass achievement in filaments. Decreased susceptibility to grazing does appears as a general feature of filaments, because filter-feeding grazers do not handle them easily (Padisak *et al.* 2003).

Zooplankton grazers selectively feed on phytoplankton cells that are of certain size (relative to their own size), which could give colonies and larger single cells an advantage, as they have fewer potential predators than small unicellulars. Supporting this idea, some authors have suggested an evolution towards extremely small sizes in the absence of grazers (Jiang *et al.* 2005). This agrees with the idea that size can be expected as a feature that differentiates unicellulars from colonies (Margalef 1978). From our data, it seems that only filaments have certain advantage not because of the size, but of the difficulty for herbivores to handle this life form in a viscous media. All in all, the highest biovolumes of filaments may result of a combination of all these suggested causes: blooming, slow sedimentation and scarce grazing.

The phylogenetic classification of organisms do not necessarily reflect ecological patterns (Salmaso & Padisak 2007), since life forms with similar requirements may have evolved in different lineages. However, if the patterns observed relating ASB, TP and life forms (Fig. 4.4.1) respond to evolutionary tendencies guided by general physical constraints rather than occasional *ad hoc* adaptations or random events, we should find similar patterns in all the main taxonomic groups holding similar life forms despite they were phylogenetically distant. In that sense, filaments showing the same ASB patterns in both Cyanobacteria and diatoms (Fig. 4.4.4) supports the hypothesis of a general physical advantage of filamentous forms under certain circumstances. Filaments in these two taxonomic groups have little in common beyond the general filamentous morphology, including contrasting sedimentation rates. Therefore, this fact reinforces the interpretation that the filamentous form may reduce grazing pressure markedly.

Contrary to the filaments case, the higher biovolumes achieved by globular colonies in chrysophytes seems to respond to phylogenetically restricted features (Fig. 4.4.4A). Although in other groups (e.g., Chlorococcales, Volvocales), globular colonies have a certain tendency for higher ASB values, they are not statistically

significant. Therefore, the high ASB values in globular chrysophytes seems to respond to an evolutionary feature of this group, perhaps related to the characteristics of these colonies that are rich in secondary metabolites that may inhibit grazing (Jüttner *et al.* 1986; Watson & Satchwill 2003; Van Donk *et al.* 2011).

4.4.3.2 Colonial life-forms species diversity across the trophic gradient

Unicellular and filamentous species distribute similarly across the trophic gradient (Fig. 4.4.3B). Therefore, it can be concluded that there have not been any particular trophic condition facilitating the diversification of filament life forms. In contrast, plate and globular colonial species distribution differ markedly from unicellulars. Globular colonies diversity follows a similar pattern than unicellulars and filaments within oligotrophy and mesotrophy, but rises faster in early eutrophy (>35 $\mu g\ L^{-1}$) than these latter forms. Plates diversify at very high TP conditions, at hypereutrophy conditions. It could be suggested that colonial forms evolved most likely in environments rich in nutrients, were they have diversified the most and, later, they have colonized more oligotrophic systems.

4.4.3.3 Cell size and colonial life-forms

In phytoplankton, growth rate have been related to cell surface and volume ratios (S/V) (Reynolds 1984). The cell surface would be proportional to the potential supply of nutrients, whereas cell volume is proportional to the demand. As S scales to L^2 and volume to L^3, being L the linear dimension of the cell, when size (L) increases, supply (L^2) will increase less than demand (L^3). Therefore, large cells will require higher external nutrient concentrations to maintain a similar specific growth rate than a small cell, all other things being equal. In other words, small size confers advantage in competition for inorganic nutrients among phytoplankton in poor-nutrient conditions (Smith & Kalff 1983).

In colonial forms, the effective surface for nutrient supply is the colony surface, not the accumulated cell surface, whereas the nutrient demand remains proportional to the accumulated cell volume. This raises the question whether the cell size

within colonies will tend to be smaller than in unicellulars, in a similar nutrient environment, to compensate for the reduced effective surface. If this is the case, the cell size decline should have to be more marked in globular colonies than in filaments, since the loss of effective surface compare to unicellulars is larger. Although there is a large variability in cell size in any life form, the smaller cells are achieved in globular colonies; from 25 to 50 % of the species have smaller cells than in the rest of life forms (Fig. 4.4.5). Furthermore, the largest cells appear exclusively related to unicellulars in all trophic states. These patterns are in agreement with a selective pressure to maintain a balance between nutrient supply, and demand to achieve the highest specific growth rate possible according to thermodynamic restrictions to metabolism.

4.4.3.4 Mucilage constraints

Many phytoplankton species are embedded in a mucilaginous matrix. Secreting mucilaginous sheaths increases algae size and thereby the prospect of an encounter with nutrient molecules in the media. Although then the diffusion media to the cells may change respect to the water, being more viscous. Occasionally, the coat may provide a high diluted media, contributing to beneficial inward diffusion gradient, if the way in the mucilage is easier than the way out. The idea that mucilages might be a repository for the concentration and storage of essential nutrients has been proposed (Lange 1976). For phytoplankton species with a known capacity to produce externally-acting phosphatases that cleave orthophosphate from organic solutes (Cembella *et al.* 1984), confining this action to inside the mucilage may result in a successful strategy. Finally, the presence of a mucilaginous coverage turns colonies and unicellulars into larger and stickier particles difficult to be ingested or less filterable by some zooplankton organisms (Lampert 1988; Hartmann & Kunkel 1991; Gliwicz 2003; Reynolds 2007). Perhaps, mucilage even may help in resisting digestion during the passage through the consumer's gut (Porter 1976).

Our data indicate that there is not a clear effect on the average species biovolume that the mucilaginous species achieve compare to similar life forms but with scarce mucilage around, despite all the potential effects of a mucilaginous coat listed above. The evolutionary trait-offs around the mucilage role have to be studied with more detail than our data provides.

5. General discussion

J.T. Bonner in "*The origins of multicellularity*" (1998) stated: "*The fact that multicellularity arose independently so many times is the primary basis for believing that there has been a significant selection for it in the ancient unicellular world. Yet it is difficult to guess what the first advantages might have been. Perhaps initially they had neither advantage nor disadvantage, and survived by drift until some further mutational change provided them with a skill that was not possible for their single-cell relatives. In early evolution becoming larger took on many forms. The most reasonable guess is that originally they arose by chance mutation, and subsequently were selected because of some advantage that they might accidentally develop.*" Can we find out the selective advantages of multicellular colonial forms in phytoplankton?

Phytoplankton embraces a large diversity of life forms. From pioneer oxygenic photosynthetic cyanobacteria to a broad spectrum of phylogenetically distant eukaryotic organisms (Falkowski *et al.* 2004). In many of the evolutionary branches colonial organisms have appeared. The evolutionary reasons for the transition to larger sizes are not yet fully understood, but multicellularity is thought to be one of its consequences (Cox & Bonner 2001). Understanding the ecological advantage that colonial forms could hold in phytoplankton was the main objective of this thesis.

Phytoplankton ecological success or failure, under certain conditions is the result of a balance between gains and losses. Unicellular and colonial organisms have to adapt their respective functional traits related to photosynthesis, resource acquisition, and predation, to changes in the environment. The advantages of a certain life-form (unicellular or colonial), could hence rely in the relevance of gain processes (light, nutrient related traits), or of losses as main drivers of phytoplankton evolution.

Despite coloniality supposes an opportunity in finding new paths to succeed, the prevalent viewpoint is that larger phytoplankton cells are worse competitors for nutrients than smaller cells and hence, they are in competitive disadvantage in nutrient-poor environments (Smith & Kalff 1982; Grover 1989). As a matter of fact, when cell size increases, the surface-to-volume ratio decreases and the average transport distance within the cell becomes larger, so diffusion becomes increasingly inadequate as a means of maintaining constant solute concentrations throughout the cytosol of the cell (Beardall *et al.* 2009). In that sense, the size of the colonies may start to become a constraint for nutrient uptake and utilization.

However, other issues that changed with size and become advantageous for colonial phytoplankton have been suggested. For example, large flagellate colonies can move and hence cover a greater space of resources likely to be exploited than small unicellulars, and also can have a larger storage capacity. Large cells (and colonies in particular) can take more advantage of the production of external enzymes since colonial forms, specially mucilaginous ones, could maintain exoenzymes close to the cells in this external matrix. In summary, any strategy by which there is not a proportional increase in the need for nutrients as body size enlarges can be regarded as a competitive advantage (Thingstad *et al.* 2005) for colonial organisms. Besides, a possible advantage for large unicellulars and colonies could be related to the top-down control of the systems by grazers, as smaller unicellulars are subject of grazing by both, small and large filter feeders, whereas large colonies can override the *edible* size spectrum of some predators (Woodward *et al.* 2005). Also the aggregation of cells to form large colonies harder to gulp or filter by zooplankton (Hessen & Vandonk 1993; Lampert *et al.* 1994) is considered a relative widespread defense strategy.

Based in the results from **chapter 1**, the review of experimental data on eco-physiological phytoplankton traits pointed to no evidence that colonial forms have an obvious advantage over unicellulars in any situation. In agreement with previous studies (Fenchel 1974; Banse 1976; Reynolds 1984; Irwin *et al.* 2006; Finkel *et al.* 2010; Edwards *et al.* 2012), and in accordance with the scaling of resource requirements to grow with cell size (Reynolds 2006), we found a constraint of size upon growth rates that irrespectively affected both unicellular and colonial organisms. Therefore, coenobia requirements, in that sense, could be comparable to those of very large cells. However, we found that phytoplankton of a certain size replicated faster as a free-living organism than as a colony, pointing to a physical constraint of the coloniality arrangement in itself. We also found that the constraint of size upon nutrient transport and diffusion within colonies cannot be avoided by enhancing the specific nutrient assimilation capacity. As a consequence, phytoplankton colonies require higher concentrations of nutrient in the media than single cells.

Specifically focused on phytoplankton P-uptake, the results of the microautoradiographic experiment of **chapter 2 also** support that higher concentrations of nutrients in the media offer less restrictive conditions for colonial

organisms. Yet they still are less competitive than smaller single-cell organisms for the nutrient up-take. P-uptake rate per unit volume was higher for the unicellular *C. reinhardtii* than for the colonial *E. elegans,* pointing to inter-specific advantages for small cells over larger colonies. *C. reinhardtii* covered in a more efficient way their P-requirements than *E. elegans* under every trophic situation. Environments richer in nutrients provide better conditions for synchronic divisions, as they reduce intra-population variability. This will facilitate coloniality too; synchronic cell division may be a requirement for certain colonial morphologies (e.g., many chlorophytes)

Phytoplankton has developed several strategies to overcome the P-limitation in certain conditions, among them, the production of exoenzymes. In **chapter 3**, we found that alkaline phosphatase activity in natural communities varied significantly among main taxonomic groups. More relevantly for the general purpose of this thesis, we found that colonial phytoplankton produced external phosphatases much more frequently than unicellulars, pointing to both: a higher constraint in P-demands and acquisition, and a possible better evolved strategy of colonies to overcome constraints imposed by size and coloniality upon nutrient uptake and utilization. In the same way, colonies of certain morphology showed comparable patterns of external phosphatases production, indicating a possible advantage of some coenobial characteristics (such as external mucilage covertures, and large intra-coenobial spaces) in the affectivity of extracellular enzyme utilization.

The results from chapters 1-3 did not pointed to any significant advantage of colonial organisms over unicellulars. In **chapter 4**, however, we found that standing biomass of filamentous phytoplankton colonies was outstandingly higher across the trophic gradient compared to other colonial and unicellular life-forms. The most likely explanation for that is a more effective predator-avoidance of these life-forms as we have not found any differential indication in favor of colonial forms related to growth traits. Therefore, the suggestion is that size and morphology in phytoplankton colonies supposes an advantage to diminish grazing, and filaments seem to be the ones with better performance, perhaps because among colonies the 1-D arrangement is the one changing the less the S/V ratio respect to isolated cells. Previous studies have shown that the limitation of picophytoplankton biomass in high-nutrient environments might be due to control

by grazers (Fogg 1986; Happeywood & Lund 1994; Irigoien *et al.* 2005). Zooplankton grazers selectively feed on phytoplankton of certain size (relative to their own size), which give colonies and larger single cells an advantage, as they have fewer potential predators than small unicellulars (Reynolds 1984). From our data, it seems that filaments have a clear advantage in the trait-off between gains and losses. The standing biomass for other colonial forms equals unicellulars, thus growth constraints of the colonies are compensated by grazing advantages, without a net advantage compared to unicellulars. However, filaments seem to reduce grazing to a higher extent than they constrain growth, thus there standing stocks achieve higher values.

The main conclusion of this thesis is, therefore, that the advantage of coloniality lies in the avoidance of grazers. Those forms that minimize the drawbacks of coloniality (e.g., filaments maximizing S/V; exoenzymes production) may have a higher yield of the grazing reduction.

Our results indicate that colonial forms have probably appeared and diversified in nutrient rich environments and, from there, colonized system across the whole trophic gradient. In accordance with some previous authors (Vermeij & Vermeij 1994; Verity & Smetacek 1996; Smetacek 2001), we suggest that defense against predation rather than growth issues have been the driving force for colonial evolution and success by competition. The study of trophic interactions on fossil records gives little information about the nature of the effect of grazers on coloniality and multicellularity in phytoplankton (Butterfield & Butterfield 2007). However, in the near future, molecular phylogenies looking for eco-evolutionary timings between phytoplankton and zooplankton may bring some light to this hypothesis.

6. Conclusions

1 Colonial organisms show the same constraints of size upon growth rates than unicellulars. As a consequence, cells of the same size grow more slowly as part of a colony than as free cells.

2 Higher nutrient concentration in the media reduces intra-population variability in nutrient uptake and softens inter-specific differences related to size. Therefore, high nutrients concentrations favor the potential synchronism of cell divisions, favoring blooms and colonial presence.

3 Phytoplankton colonies in nutrient rich environments suffering P imbalance produce much more external alkaline phosphatases than unicellular organisms of any taxa. It can be argued that the colonial form has some advantages for obtaining a higher yield of exoenzymes (e.g., cell proximity, mucilaginous envelops, spherical empty morphologies).

4 Filaments achieve higher population biovolumes across the entire trophic gradient than any other colonial form and unicellulars. It is suggested that this is a better yield between grazing and growth reduction than in other life forms.

5 Defense against predation rather than growth issues seems to have been the driving force for colonial evolution in phytoplankton. Nutrient rich environment minimizes the nutrient supply constraints inherent to coloniality and probably imposes higher grazing pressures, therefore, it is suggested that colonial forms will tend to evolutionary appear in those environments, particular under high grazing pressure, and then spread through oligotrophic conditions.

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8. Annexes

Annex I

List of species and experimentally determined traits corresponding to the bibliographic survey used in Chapter 1

V: Cell volume (µm3) calculated according Annex II; **L**: Cell length (µm); **W**: Cell width (µm); **Sr**: Sinking rate (µm s^{-1}); **Vs:** Sinking velocity according to Stoke's formula (µm s^{-1})**(**)**; **Gr**: Growth rate (day^{-1}); $\mathbf{T_{opt}}$: Optimal growth temperature (°C); $\mathbf{I_{opt}}$**:** Optimal light intensity (µmol photons m^{-2} s^{-1}); $\mathbf{P_{max}}$: Maximal production rate (mgC $mgChla^{-1}$ h^{-1}); $\mathbf{K_m}$**:** Half-saturation constant for uptake of nutrients (NO_3^{2-}, PO_4^{3-}, NH_4^+**).**

Unicell species	V (*)	L (*)	W (*)	Sr (1)	Vs (*)	Gr (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
***Actinocyclus* sp.**				6.94									(1)[1]
Ankyra judayi						1.03	20						(3,4)[2]
***Carteria* sp.**	2532	23	15		2.49		20				1.41		(8)[3] (6) [4]
***Chlamydomonas* sp.**										0.0071			(7)[5]
C. reinhardtii	2010.6	16	15	2.2	2.14	3.8	25	95			2.39		(3) [6] (1) [7] (5,6)[8] (8) [3]
***Chlorella* sp.**										0.022			(7) [5]
C. vulgaris	220.89	7.5	7.5	0.438	0.489	1.3	30	140		110.53	0.89	331.58	(1) [9] (6)[10]
C. pyrenoiodosa											0.25		(8)[3]
***Chlorococcum* sp.**	3473.6	19	19	8.17	3.08	1.59					4.14		(3)[11] (1)[9] (8)[3]
Chromulina freiburgensis	231.9	12	7.5		0.51	2.66							(3)[12]
***Chroomonas* sp.**						0.85		116			0.4		(3)[13] (8)[14]
Closterium sp.				5.78									(1)[15]
***Coscinodiscus* sp.**	1164.2	16	39		1.48	0.695	20			0.045			(3,4)[16] (7)[17]
C. radiatus				25.46									(1)[18]
C. wailesii						0.695	21			5.62	5.1	5.5	(3)[16] (7)[19] (8,9)[20]
C. rex									1.15				(8)[21]
C. lineatus											2.8	2.8	(8,9)[20]
Cosmarium abbreviatum	2766	17	18		2.64	0.48	20			12.44			(3,4,7)[22]
C. subprotumidum	8485.8	27	25		5.58	1		400					(3,5)[23]
C. botrys	143659	77	60	17	36.78								(1)[24]
***Cryptomonas* sp.**						0.85	9.2	116	1.5	0.014			(3)[13] (4,6)[25] (7)[5]

Unicell species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Cryptomonas erosa	1772	24	12	3.59	1.96	0.85	15				3.9		(1)[26] (3,4)[13] (8)[27]
C. marsonii	1520.5	24	11	3.7	1.77								(1)[26]
C. ovata	4424.4	50	13		3.61	0.86	15	198					(3,4,5)[13]
C. phaseolus	397.6	13	7.5		0.73	0.8	15	92					(3,4,5)[13]
C. rostraformis	12469	54	21		7.21	0.39	15						(3,4)[13]
Cyclotella meneghiniana	452.38	3.4	3.35	0.96	0.79	0.34	16				0.005		(3,4)[28] (1)[29] (8)[30]
C. nana						3.4	20			0.011	0.7	0.4	(3,4)[28] (8,9)[20] (7)[31]
C. lomerata	23.76	5.5	5.5		0.11	2.04			6.7				(3,6)[32]
C. cryptica	93.31	11	10.9		0.28	0.61							(3)[33]
Diatoma tenuis						0.46							(3)[34]
D. elongatum										2.74			(7)[29]
***Dunaliella* sp.**	606.13	19	10.5		0.96								
D. tertiolecta	448.9	6	9.5	69.44	0.79	2.32	25	500	2.95		11.1	25	(3,4,5)[20] (1)[35] (6)[21] (5,7,8,9)[36]
Gomphoneis minuta									3.264				(5)[37]
***Golenkiniopsis* sp.**											4.49		(8)[3]
Haematococcus pluvialis	12770	29	29		7.33	1.2	23	9.67					(3,4)[28] (5,6)[4]
Monochrysis lutheri	107.53	5.9	5.9	2.083	0.30	1.5	15		2.94		0.6	0.5	(3,4)[28] (1)[20] (6)[21] (8,9)[20]
Monodus subterraneus						0.93	25						(3,4)[28]
***Monoraphidium* sp.**	56.3	16	3.7	0.07	0.20		20				1.56		(1)[15] (8)[3]
M. minutum	48.8	11	4.1		0.178	2.09	20						(3,4)[2]
***Navicula* sp.**				20.83							0.24		(1)[38] (8)[39]

Unicell species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Navicula minima	37.38	11.5	3.25		0.15	1.4							(3)[28]
N. incerta							25	800	0.3				(4,5,6)[40]
N. pelliculosa	54.83	10.75	51	0.194							6.94		(8)[3]
***Nitzschia* sp.**				20.83		1.31	25	60	7.77	2.64			(3,5)[41] (1)[38] (5)[42] (7)[3]
N. frigida						0.4							(3)[34]
N. acicularis	324	90	3.6		0.63					0.0023			(7)[5]
N. linearis	655	131	5	1.011						0.019			(7)[5]
N. palea	106.25	42.5	2.5		0.301					0.047			(7)[5]
Phacotus lenticularis						0.74	20					0.55	(6)[4] (3,9)[2]
***Rhizosolenia* sp.**	2613.8	39	16		2.54	0.13	20	50	$2_{x10^{-6}}$				(6)[44] (3)[45]
R. delicatula											0.93		(8)[43]
R. acuminata						0.78							(3)[46]
R. formosa						0.37			0.28				(3)[46] (6)[45]
R. castracanei						0.38							(3)[46]
R. imbrincata				72.92									(1)[47]
R. setigera				20.49									(1)[47]
R. tolterfothii											1.7	0.5	(8,9)[20]
R. robusta											3.5	9.3	(8,9)[48]
***Rhodomonas* sp.**	53	7.75	4.5	0.81	0.189								(1)[26]
R. carterae						0.39							(3)[33]
R. salina						0.64							(3)[33]
R. minuta	112.9	11.5	5.5	0.81	0.31	0.95	20				0.26		(1)[26] (3,4,8)[2]
R. salina						0.64	20						(3,4)[33]
R. minuta	112.9	11.5	5.5	0.81	0.31	0.95	20				0.26		(1)[26] (3,8)[2]
Staurastrum pingue						0.77	27	270					(3,4,5)[49]
S. chaetoceros						0.48				6.95			(3,7)[50]

Unicell species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Staurastrum furcigerum	34682	41.5	56.5	3	14.26								(1)[24]
Stephanodiscus minutulus	96.21	2.5	7		0.28	1.24	20						(3,4)[51]
S. binderanus	153.93		14	9.26	0.39	0.59	20						(1) [52]
S. hantzschii	538.78	3.5	14	0.38	0.89								(1)[52]
S. neoastraeae	11545	1	35		6.85			615.7	8.724				(5, 6) [53]
***Synechococcus* sp.**				0.03		9.9	45	60	2.7	0.2			(3)[28] (4, 5, 6) [54] (1)[55]; (7)[56]
S. lividus						8.5	52						(3, 4) [28]
Synechocystis minima						1.32	32						(3, 4) [49]
Synedra acus	550	200	5.5	7.3	0.900	1.061	20						(3, 4) [51] (1)[57] ;
S. ulna	700	200	7		1.057								
S. radians										0.00014			(7)[5]
S. rumpens										0.0069			(7)[5]
Tetraedron minimum	40.5	9	4.5		0.158								
***Thalassiosira* sp.**				7.99							53		(1)[18] (8)[58]
T. decipiens				4.63									(1)[18]
T. rotula	706.85	12	30	26.62	1.064	2.4	25						(1, 3, 4) [59]
T. pseudonana				0.87		1.29	25		7.58	0.0034	2.2		(6)[32] (3, 4, 8) [33] (7) [17]
T. weissflogii						0.75					2.8		(3, 8) [33]
T. baltica						0.55	4						(3, 4) [34]
T. levandera						0.63	4						(3, 4) [34]
T. fluviatilis										0.0011			(7)[60]

Colonial species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I opt (5)	P max (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Actinastrum hantzschii	632	17.5	4.5		0.99		20	172			10.67		(5,6)[25] (8)[3]
***Anabaena* sp.**				5.44				53					(5)[61] (1)[62]
A. flos-aquae	3120	8	5	5.44	2.86		20			2.8	0.08	2.22	(1, 4)[57] (7)[25] (8)[63]
A circinalis	1005	8	4		1.35				5.02				(5)[64]
A oscillarioides										0.71			(7)[65]
A spiroides	5400	7	7	1.15	4.13								(1) [26, 66]
A variabilis	600	4.6	5		0.95	1.15				1.1			(3, 7) [67]
Anabaenopsis sp.									1.52				(6)[68]
Ankistrodesmus falcatus	21811	100	7		10.4	1.3	35	148			1.2		(3, 4, 5) [69]
***Aphanizomenon* sp.**				0.46		1.56	19	35		1.5			(4, 5, 7) [70] (1)[71] (3, 6) [72]
A flos-aquae	2620	10	5		2.55	0.78	20			28.71			(3, 6, 7) [73]
***Apahanocapsa* sp.**						1.104							(3)[74]
Aphanothece clathrata									1.63				(6)[75]
A. microscopica						0.816	35						(3, 4) [76]
Arthrospira platensis	1232	4	7		1.54	0.41	30	180					(3, 4, 5) [77]
A..maxima						2.536					20.4		(3, 8) [78]
Asterionella formosa	2080	130	2	5.78	2.18	1.74	20	40					(1, 3, 4, 5) [57]
A. japonica											1.3	1.5	(8,9)[20]
Aulacoseira subartica	2356	9	6	7.4	2.37	0.214	30	819	5.28	0.021	1.77		(3)[79] (4, 5, 6) [80] (7, 8) [81]
Botryococcus braunii						0.21	25	200					(3)[82] (4, 5) [83] (6)[10]
Bumilleriopsis brevis	1005	20	4		1.35	2.9	19						(3, 4) [28]
B. filiformis	22619	50	12		10.72				5.88				(6)[84]
***Chaetoceros* sp.**										2.6	3.1		(7)[19]; (8)[85]
C. radicans				2.31									(1) [18]

Colonial species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Chaetoceros wighamii						0.8	18						(3, 4) [86]
C. affinis						1.57			5.15				(3, 6) [32]
C. curvisetum						0.672				0.944	1.7	16.5	(3, 8, 9) [87] (7)[88]
C. gracilis										0.039	0.3	0.5	(3)[20] (7)[60] (8, 9) [89]
Chroococcus minutus	880	7.5	7.5		1.23	0.36							(3)[90]
Chrysosphaerella sp.									0.7				(6)[91]
***Coccomyxa* sp.**						0.5	25	45	0.87				(4, 5, 6) [92] (3)[93]
Coelastrum microporum	35340	15	15		14.4	1.64	35	420					(3, 4, 5) [23]
***Cyanobium* sp.**						1.52	15	10	3.06				(3, 4) [94]; (6)[95]
Cylindrospermum psisraciborskii	38160	180	3							51.27			(7)[73]
C. sphaerica						0.17	25						(3, 4) [28]
Detonula confervacea						1.4	10						(3, 4) [28]
***Dictyosphaerium* sp.**						2.8	25	172					(3,4,5) [96]
Dinobryon divergens						0.72	17	60	0.42				(3)[97] (4)[91] (5, 6) [98]
Eremosphaera viridis							25	1000	0.13				(4, 5, 6) [99]
Eucampia zodiacus				13.19		3.08	20			1.83	2.59		(1)[18](3, 4, 5, 7, 8) [19]
Eudorina elegans	33504	20	20		13.9	0.62	23	172					(6)[25]
***Fragilaria* sp.**	9000	150	3	11.19	5.8								(1)[57]
F. pinnata	1900	19	5		2.06						1.64		(8)[3]
F. crotonensis	9900	150	3	11.2	6.18	0.58	25	150			0.85		(6)[25] (8)[3](3, 4, 5) [49];
***Gloebotrys* sp.**						0.144							(3)[101]
Gloeocapsa alpicola						1.92	30	165	1.8		5.7		(3, 4, 5, 6) [102]; (8)[3]
***Gloeothece* sp.**						0.384	26	80					(3, 5) [103]
Gomphosphaeria aponina	**3617.4**	9	14		3.15	0.942							(3)[100]
G. lacustris	314	4	3	1.27	0.6								(1)[66]

Colonial species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I opt (5)	P max (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Guinardia flaccida				11.92			11		1.95				(1)[18](5, 6) [104]
G. striata						1.04							(3)[105]
Homoeothrix crustcea						3.42							(3)[106]
Lauderia annulata				8.8		0.97							(1)[18] (3)[107]
Leptocylindrus danicus							20				1.3	3.4	(4, 8, 9) [20]
Lyngbya wollei							26	11					(4, 5) [108]
***Melosira* sp.**	125660				33.6								(6)[25]
M. granulata	6636.6	25	13	7.43	4.74	0.03		172					(3)[109] (1, 5)[57]
M. arctica						0.64							(3)[34]
Merismopedia tenuissima	44.8	1.75	1.75		0.17	0.99	30	6.14	7.06				(3, 4, 5, 6) [80]
Micractinium pusillum	1046.4	5	5		1.38	0.58	35	182					(3, 4, 5) [110]
***Microcystis* sp.**	65000	5	5		21.68						2.2		(8)[111]
M. aeruginosa	65450	5	5	8.1	21.78	0.48	23	436	6.97	0.25			(3)[57] (4, 5, 6) [80] (1)[112] (7)[73]
Mougeotia thylespora				1.16						0.024			(1)[113] (8)[114]
Nodularia spumigena	9850	8	5.6		6.16	0.149	30	30			0.02		(3, 4, 5) [115] (8)[116]
***Oocystis* sp.**							35	120	1.848				(4, 5, 6) [117]
O. borgei	7344	14.5	11		5.07	1.6	35	120					(3)[86]
O. rhomboidea	122.4	6.5	3	0.016	0.33								(1)[118]
O. pusilla	601.6	9								0.012			(7)[5]
O. marsonii	1858.4	10.5	6.5		2.03	0.72	20				1.01		(3, 4, 8) [2]
Oscillatoria agardhi	1178	3	5	1.50	0.138	10					16		(1, 3, 4) [119] (8)[120]
O. thiebautii										9			(7)[121]
O. redekei	529.2	11	1.75		0.877	0.126	6	89					(3, 4, 5) [69]
Pandorina morum	16362.4	12.5	12.5	0.93	8.64	0.01						0.5	(1)[113](9)[2]
Pediastrum duplex	152909	27	13		38.34	0.21							(3)[122]
***Phormidium* sp.**						1.59	35	426					(3, 4, 5) [69]
P. laminosum		3	1.25							3.4			(7)[123]
P. uncinatum	3534.2	4	7.5		3.11						300		(8)[124]

Colonial species	V (*)	L (*)	W (*)	S_r (1)	V_s (*)	G_r (3)	T_{opt} (4)	I_{opt} (5)	P_{max} (6)	PO_4 K_m (7)	NO_3 K_m (8)	NH_4 K_m (9)	Ref.
Planktosphaeria gelatinosa	30536	18	18		13.10	0.13							(3)[122]
Plankthotrix agardhii								483	6.276				(5, 6) [53]
P. rubescens				5.9		0.11		420					(1, 3, 5) [125]
Scenedesmus acutus	188	10	3	3.52	0.44								(1)[126]
S. protuberans		2.66	5.75			1.32	20	117.52	1.428				(3, 4, 5) [127] (6)[128]
S. obliquus	332	10	4		0.64					0.016	1.1		(7)[60] (8)[3]
S. acutiforme	994.8	15.7	5.5	1.15	1.34								(1)[66]
S. quadricauda	872	13	4			1.22				0.035		0.0083	(9)[67] (7)[5] (3)[57]
Skeletonema costatum				4.98		0.6	10	40	6.2	0.68	0.4		(1)[18](3, 4, 5) [80] (6)[129]
Snowella lacustris				1.04		0.9	18						(3, 4) [86] (1)[57]
Sphaerocystis schroeteri	36810	13	13		14.84	0.79	20			0.025		0.64	(3, 4, 9) [2] (7)[5]
***Synura* sp.**						2.04		60	3				(3)[32] (5, 6) [98]
Tabellaria flocculosa	32000	50	5	10.26	13.52	0.76	20	172					(1, 3, 4) [57] (5)[25]
Thalassionema nitzschoides	3060	60	3	9.03	2.83	1.9	31						(3, 4) [130]
***Tribonema* sp.**				24.24		0.53	17						(3, 4) [97] (1)[15]
T. monochloron	8207.4	10	5.5		5.46		14	4.56	13.6				(4, 5, 6) [131]
***Trichodesmium* sp.**											0.4		(8)[132]
Trichodesmium thiebautii												6.74	(9)[121]
Tychonema bourellyi						0.76	17						(3, 4) [97]
***Ulothrix* sp.**								1200					(5)[37]
***Uroglena* sp.**							9.5		2.7				(4, 6) [91]
Uroglenopsis americana	4578	5	5		3.70	0.01							(3)[109]
Volvox aureus	120250	5	5		32.67	0.69	20						(3, 4) [133]
V. globator	240329	3	3		51.83	0.43	20						(3, 4) [133]

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Annex II

Formulae for organisms' volume calculation

Volume Shape	*Formulae*	*Taxon Examples*
Circle based cylinder - long	π*L*D*D/4	*Aphanizomenon, Aulacolseira*
Circle based cylinder - short	π*H*D*D/4	Centric diatoms
Circle based ellipse	π*L*D*D/6	
Oval based cylinder	π*L*D*H/4	
Oval based ellipse	π*L*D*H/6	
Cone	π*L*D*D/12	*Mallamonasakrokomos*, horn of *Staurastrum*
Cone + hemisphere	(π*D*D)12*(D/2+L)	*Rhodomonas, Mallamonascaudata*
Double cone	π*L*D*D/12	*Ankistrodesmus, Closterium*
Cuboid/rectangle	L*D*H	*Tabellaria*, pennate diatoms, *Merismopedia*
Cuboid/rectangle * 0.5	0.5*L*D*H	*Nitzschiaacicularis*
Sphere	π*D*D*D/6	*Microcystis, Sphaerocystis*, picoplankton cells
0.2 sphere	0.2*π*D*D*D/6	*Woronichinia*
0.25 sphere	0.25*π*D*D*D/6	*Eudorina*
0.5 sphere	0.5*π*D*D*D/6	*Aphanothece, Aphanocapsa*
0.75 sphere	0.75*π*D*D*D/6	*Snowella, Gomphosphaeria*
Microcystis colony	Average cell volume* nº cells in colony where nº cells in colony (y) from logy=2.99log10x-2.88 and where x = diameter (D) of colony	*Microcystis*colony calculation from (Reynolds, 1984)
Staurastrum	(P*2)*π*L*D*D/12	*Staurastrum*
L, Length (µm); D, Diameter or width (µm); H, Depth or height (µm); P, nº arms/branches in *Staurastrum* half cell		

Annex III

Potthoff method: comparing regression lines from independent samples

The analysis discussed in this document is appropriate when one wish to determine whether the linear relationship between one continuously distributed criterion variable and one or more continuously distributed predictor variables differs across levels of a categorical variable.

In the simplest case, a Potthoff analysis is essentially a multiple regression analysis of the following form:

$$Y = a + b_1C + b_2G + b_3C*G$$

Where Y is the criterion variable, C is the continuously distributed predictor variable, G is the dichotomous grouping variable, and C*G is the interaction between C and G.

Test of Coincidence

A Potthoff analysis starts with a test of the null hypothesis that the regression line for predicting Y from C is the same at all levels of some grouping variable. To test this null hypothesis of coincident regression lines, we compare the full model containing the continuous predictor, the grouping variable, and the interaction) with a model that contains only the continuous predictor.

Linear regressions for the full model and the model containing only the continuous predictor are performed, and R^2 compared to seek for differences. A partial *F*-test is then calculated to check if these differences are large enough to be statistically significant.

$$F = \frac{SS_{reg-full} - SS_{reg-reduced}}{(f-r)MSE_{full}}$$

Where f is the number of predictors in the full model, r is the number of predictors in the reduced model. MSE_{full} is the mean square error of the most complete model, and $SS_{reg\text{-}full}$, $SS_{reg\text{-}reduced}$, are the sum of squares of the most complete model and the reduced model respectively. The numerator degrees of freedom is (f-r), the denominator degrees of freedom is (n-f-1). Non-coincident lines may differ in slope and/or in intercept.

Test of Parallelism

To test the null hypothesis that the slope throughout the levels of the grouping variable, we need to determine whether or not removing the interaction term, C*G from the model significantly reduces the R^2. That is, we need to compare the full model with a reduced model containing all the terms but the interaction, by carrying out another partial *F*-test. This partial *F*-test is equivalent to that called the "Test for Parallelism" by Kleinbaum and Kupper (1978) (Kleinbaum & Kupper 1978), and called the "Common B-Coefficient test" by Potthoff (1966).

Test of Intercepts

If statistically significant result has been obtained so far, the lines would have already been shown to differ in slopes, but we wouldn't have yet tested for a difference in intercepts. To test the null hypothesis that the intercepts are identical across groups, we need to compare the full model with a model that does not contain the dichotomous grouping variable, again with a partial *F*-test, that allows us to conclude, if the intercept for predicting our response is the same for the levels of our groups.

Assumptions of the Potthoff technique

As with other multiple regressions, to use *t* or F statics we must assume that the error component is normally distributed and that error variance is constant across groups. If you have heterogeneity of variance, you should consult Kleinbaum and Kupper (1978)(Kleinbaum& Kupper 1978) for large sample z-tests that don't pool error and for a reference to a discussion of other alternatives.

Kleinbaum D.G. & Kupper L.L. (1978). *Applied regression analysis and other multivariable methods.*, Boston: Duxbury.

Potthoff R.F. (1966). *Statistical aspects of the problem of biases in psychological tests.*, Chapel Hill: University of North Carolina, Department of Statistics. .